- 5 -

Remarks

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

Nonelected claims 18-31 have been cancelled without prejudice, claims 1, 2, 7, 9, 11, 16, and 17 have been amended. Descriptive support for the amendment to claim 1 is provided at paragraphs [0004]-[0007], [0035], and [0069], which discuss symptoms or conditions associated with interstitial cystitis and pelvic pain disorders. Therefore, no new matter is introduced by these amendments.

Claims 1-17 are pending, and no excess claim fees are due with this submission.

This submission is accompanied by (i) a petition for three-month extension of time, (ii) an Information Disclosure Statement and copies of two references; and (iii) a request for a corrected filing receipt (for correction of a typographical error in the title). All fees associated therewith should be charged to deposit account 14-1138, and any overpayment/underpayment should be credited/charged to this same account. The return of a signed and initialed IDS with the next office communication is respectfully requested.

The rejection of claims 1-17 under 35 U.S.C. § 112, first paragraph, for lack of enablement is respectfully traversed.

At pages 2-6 of the office action, the U.S. Patent and Trademark Office ("PTO") asserts several bases for this rejection.

The first basis concerns the scope of the method with respect to pelvic pain syndromes generally, whereas the data in the application concerns the detection of interstitial cystitis. It is well-established that cross-talk exists between different pelvic organs, whereby stimulation of non-bladder afferents produces measurable affects on the bladder, and *vice versa*. Pezzone *et al.*, "A Model of Neural Cross-Talk and Irritation in the Pelvis: Implications for the Overlap of Chronic Pelvic Pain Disorders," *Gastroenterology* 128:1953-1964 (2005) ("Pezzone")(copy attached as Exhibit 1) confirms that irritable bowel syndrome, interstitial cystitis, and other chronic pelvic pain disorders often occur concomitantly, and provides "compelling evidence" that afferent irritation of one pelvic organ can adversely influence and sensitize another pelvic organ via neural interactions (*see* abstract). Pezzone specifically demonstrates cross-talk between the colon and lower urinary tract. *Id.* A subsequent publication implicates mast cell infiltration as well as urinary bladder C fibers and the release of their active neuropeptides in the pelvic cross-sensitization process (Ustinova *et al.*, "Sensitization of the

Pelvic Nerve Afferents and Mast Cell Infiltration in the Urinary Bladder Following Chronic Colonic Irrigation is Mediated by Neuropeptides," *Am. J. Renal Physiol.* 292:F123-130 (2007)(Ustinova")(copy attached as Exhibit 2)(*see* abstract).

Consistent with the examples in the present application, demonstrating a statistically significant association between calcitonin gene-related peptide ("CGRP") levels and interstitial cystitis, Sarna, "Enteric Descending and Afferent Neural Signaling Stimulated by Giant Migrating Contractions: Essential Contributing Factors to Visceral Pain," *Am. J. Physiol. Gastrointest. Liver Physiol* 292:G572-581 (2007)("Sarna") (copy attached as Exhibit 3) demonstrates that close intra-arterial infusion of CGRP to the proximal intestinal segment can produce changes in heart rate just like bowel distension, replicated mechanically by Sarna with a balloon (*see* page G574, Figs. 1C-D and 3). Balloon distension is frequently used to induce bowel pain (measured by heart rate), and both the balloon distension and CGRP administration afforded measurable bowel pain. Delafoy *et al.*, "Interactive Involvement of Brain Derived Neurotrophic Factor, Nerve Growth Factor, and Calcitonin Gene Related Peptide in Colonic Hypersensitivity in the Rat," *Gut* 55:940-945 (2006) (copy attached as Exhibit 4) confirms that CGRP is implicated in several models of visceral pain (*see* page 940) and demonstrates that the CGRP antagonist (peptide $CGRP_{8-37}$) inhibited colonic pain hypersensitivity (*see* page 942, Fig. 1; page 943, Fig. 4). Bourdu *et al.*, "Rectal Instillation of Butyrate Provide Novel Clinically Relevant Model of Noninflammatory Colonic Hypersensitivity," *Gastroenterology* 128:1996-2008 (2005)(copy attached as Exhibit 5) confirms that the CGRP receptor antagonist (peptide $CGRP_{8-37}$) inhibits colonic pain hypersensitivity and suggests that CGRP receptors provide "a promising target for treatment of IBS" (*see* abstract).

Because of the substantial cross-talk between pelvic organs and the demonstrated association of CGRP in visceral pain involving these organs (e.g., bowel and bladder), persons of skill in the art would fully expect that CGRP can be used as a measure of pelvic pain syndromes generally as well as in interstitial cystitis particularly.

The second basis of rejection concerns the presence of data to demonstrate practice of the claimed invention. As noted above, the examples of the present application include data illustrating the association of CGRP with interstitial cystitis as measured by ELISA. The PTO discounts the working examples, asserting that there is no clear difference between interstitial cystitis patients and control patients, because the CGRP levels for these two groups partially overlap. Applicants believe this is improper. The examples in the present application

demonstrate that a *statistically significant* relationship exists between bladder CGRP levels and interstitial cystitis. This was confirmed by Mann Whitney non-parametric test ($U=105$, $p<0.01$); and the one-way analysis of variance was also significant ($F_{(1,21)} = 6.85$; $p=0.0161$). Thus, the examples clearly support the use of CGRP levels as a statistically significant indicator for interstitial cystitis. Because the interstitial cystitis patients had been previously diagnosed (i.e., they clearly had symptoms of interstitial cystitis beyond the ELISA results), persons of skill in the art would recognize that the combination of elevated CGRP plus the symptoms can be used to diagnose interstitial cystitis. Therefore, the statistical significance of the data presented in the application cannot be summarily ignored for the reasons asserted by the PTO.

The third basis of rejection concerns the PTO's perception that the prior art teaches away from any association between CGRP or PACAP and interstitial cystitis (citing Kreder *et al.*, Urology ICBR-69 57(6A):128-9 (2001) and Vizzard, *J. Comp. Neurol.* 420:335-48 (2000)). As demonstrated above, a statistically significant association exists between CGRP levels and interstitial cystitis. That the prior art might have taught away from the presently claimed invention does not negate the enablement afforded by presented examples.

For these reasons, the rejection of claims 1-17 should be withdrawn.

Respectfully submitted,

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Exhibit 1: Pezzone *et al.*, "A Model of Neural Cross-Talk and Irritation in the Pelvis: Implications for the Overlap of Chronic Pelvic Pain Disorders," *Gastroenterology* 128:1953-1964 (2005)

A Model of Neural Cross-Talk and Irritation in the Pelvis: Implications for the Overlap of Chronic Pelvic Pain Disorders

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Background & Aims: Irritable bowel syndrome, interstitial cystitis, and other chronic pelvic pain (CPP) disorders often occur concomitantly. Neural cross-talk may play a role in the overlap of CPP disorders via the convergence of pelvic afferents. We investigated the hypothesis that afferent irritation of one pelvic organ may adversely influence and sensitize another via neural interactions. *Methods:* We measured pelvic organ smooth muscle and striated muscle reflexes during micturition and colorectal distention (CRD) in urethane-anesthetized rats. The effects of acute cystitis on distal colonic sensory thresholds to CRD and the effects of acute colonic irritation on micturition parameters were assessed. *Results:* External urethral sphincter (EUS) electromyography (EMG) was typical for the rat, with phasic firing during micturition. External anal sphincter EMG also showed phasic firing during micturition in synchrony with EUS activity but, in addition, showed both tonic bursts and phasic firing independent of EUS activity. Before bladder irritation, graded CRDs to 40 cm H_2O produced no notable changes in abdominal wall EMG activity. Following acute bladder irritation, dramatic increases in abdominal wall EMG activity in response to CRD were observed at much lower distention pressures, indicating colonic afferent sensitization. Analogously, following acute colonic irritation, bladder contraction frequency increased 66%, suggesting sensitization of lower urinary tract afferents. *Conclusions:* We report compelling evidence of bidirectional cross-sensitization of the colon and lower urinary tract in a novel experimental model. This cross-sensitization may account for the substantial overlap of CPP disorders; however, further studies are needed to fully characterize these pathways.

Chronic pelvic pain (CPP) encompasses a group of debilitating disorders primarily affecting women of reproductive age. Characterized by pain involving the pelvic cavity (irritable bowel syndrome [IBS] and interstitial cystitis [IC]) and/or the pelvic floor (levator ani syndrome, urethral syndrome, prostatodynia, vulvodynia, and orchialgia),[1] CPP affects as many as 15% of women

in both the United States and the United Kingdom.[2,3] Because the colorectum and urinary bladder are two of the larger pelvic organs and because their functions are an integral part of daily, conscious, physiologic pelvic activity, it is perhaps not surprising that IBS and IC, analogous disorders of pelvic visceral pain and urgency, are two of the more common manifestations of CPP.

IBS, an intestinal disorder characterized by chronic or recurrent lower abdominal pain or discomfort associated with altered stool consistency and frequency,[4] is the most common gastrointestinal cause of CPP, affecting 50% of such women presenting to gynecologic clinics.[5–8] Patients with IBS, 70% of whom are women, incur 74% more direct health care costs than those without IBS and have more physician visits for both gastrointestinal and nongastrointestinal symptoms.[9–11] IBS alone results in an estimated $8 billion in direct medical costs annually.[12] IC or painful bladder syndrome, a CPP disorder that afflicts women almost exclusively, is characterized by unpleasant urinary symptoms such as urinary frequency, urgency, nocturia, and, most notably, pain (suprapubic, pelvic, urethral, vaginal, and perineal) related to bladder filling in the absence of active infection or organic disease.[13,14] More than 700,000 women in the United States have IC,[15] and associated yearly direct and indirect costs exceed $430 million according to 1982 figures.[15] Health care costs aside, IC, IBS, and other causes of CPP impact dramatically on quality of life, and their predilection for women also adds to the health burden of this poorly studied population.

Although the etiologies of both IBS and IC have been studied extensively (albeit mutually exclusively) and their prevalence is frequently concurrent, few have con-
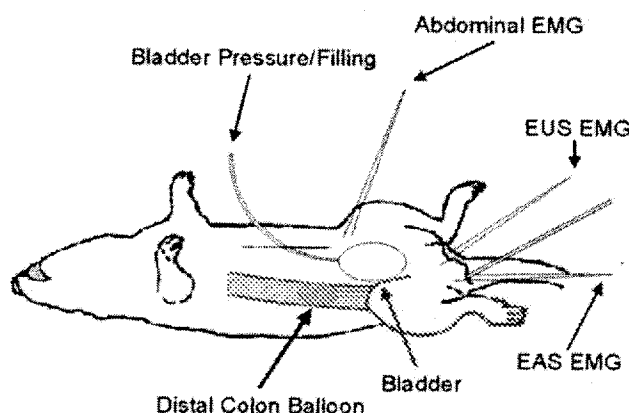

Figure 1. In vivo physiologic instrumentation.

sidered a common underlying mechanism responsible for the development and the overlap of these and other causes of CPP. As many as 40%–60% of patients diagnosed with IBS also exhibit symptoms and fulfill diagnostic criteria for IC; correspondingly, 38% of patients diagnosed with IC also have symptoms and fulfill diagnostic criteria for IBS.[6,16,17] Furthermore, 26% of patients diagnosed with IC have also been found to have concurrent pain of the vulva or vulvodynia,[18] and 45% of men with chronic prostatitis or male CPP exhibit pain with bladder filling, a classic feature of IC.[19] The high concurrence rate of IBS, IC, and other CPP disorders is therefore substantial and may suggest a common predisposition, a shared etiologic factor, or possible cross-sensitization of pelvic organs.

Neural cross-talk in the pelvis, which occurs when afferent activation of one pelvic structure influences efferent output to another, is necessary for the normal regulation of sexual, bladder, and bowel function and is likely mediated by the convergence of sensory pathways in the spinal cord.[20–25] For example, overlapping central projections of pelvic and pudendal afferents allow integration of somatic and parasympathetic motor activity in the pelvis and facilitate the orchestration of sacral reflexes. Correspondingly, the convergence of afferents from the bladder and bowel is a common feature of visceral interneurons that are believed to mediate vesicosphincteric and colono-sphincteric reflexes and colonovesical cross-inhibitory interactions.[26] Because a neural substrate for pelvic organ cross-talk exists under normal conditions, alterations in these neural pathways by disease or injury may play a role in the development of overlapping CPP disorders and pelvic organ cross-sensitization.

Previously, no one has adequately investigated the hypothesis that afferent sensitization of one pelvic organ may adversely influence and sensitize the other via direct neuronal connections, reflexes, or changes in central pro-

cessing. We hypothesized that acute irritation of one pelvic organ could lead to afferent sensitization of another via shared pelvic afferent innervation and/or convergent afferent pathways and that this cross-sensitization could account for an overlap of CPP disorders and result in "referred" pelvic pain and possibly neurogenic inflammation. To shed light on these issues, we have developed a rodent model for studying pelvic organ reflexes, pelvic organ cross-talk, and associated striated sphincter activity that has allowed us to show that (1) colonic afferent sensitization occurs following the induction of acute cystitis and (2) urinary bladder sensitization occurs following the induction of acute colitis.

Materials and Methods

Animals

Female Sprague–Dawley rats, 200–250 g in weight, were purchased from Hilltop Lab Animals, Inc (Scottsdale, PA) and housed in standard polypropylene cages with ad libitum access to food and water in the University of Pittsburgh's Central Animal Facility. All studies were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee and were found to meet the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

General Experimental Paradigms

In the first series of experiments, anesthetized female rats (n = 8) underwent placement of bladder cystometry catheters and intracolonic balloons. Abdominal electromyography (EMG) measurements were obtained during saline cystometry both before and after acute bladder irritation with protamine sulfate and KCl. In the second phase of experiments, a second set of anesthetized animals (n = 4) underwent placement of bladder cystometry catheters and underwent cystometric measurements before and after acute colonic irritation with trinitrobenzene sulfonic acid (TNBS).

In Vivo Physiologic Instrumentation

Urinary bladder measurements. Female rats were first anesthetized with urethane (Sigma Chemical Co, St. Louis, MO) 1.2 g/kg subcutaneously; following a midline laparotomy, a transvesical, flared-tipped PE-50 catheter (Fisher Scientific, Hanover Park, IL) was inserted through the bladder dome via a small cystotomy and ligated for urinary bladder filling and pressure recording. The catheter tubing was externalized via the proximal aspect of the ventral abdominal incision and connected to a blood pressure transducer (World Precision Instruments, Sarasota, FL) and a syringe pump (Harvard Apparatus, Holliston, MA) via 3-way stopcocks. Normal saline was infused into the bladder at a rate of 0.1 mL/min for continuous open cystometry. A Transbridge transducer amplifier (World Precision Instruments) was used to amplify the
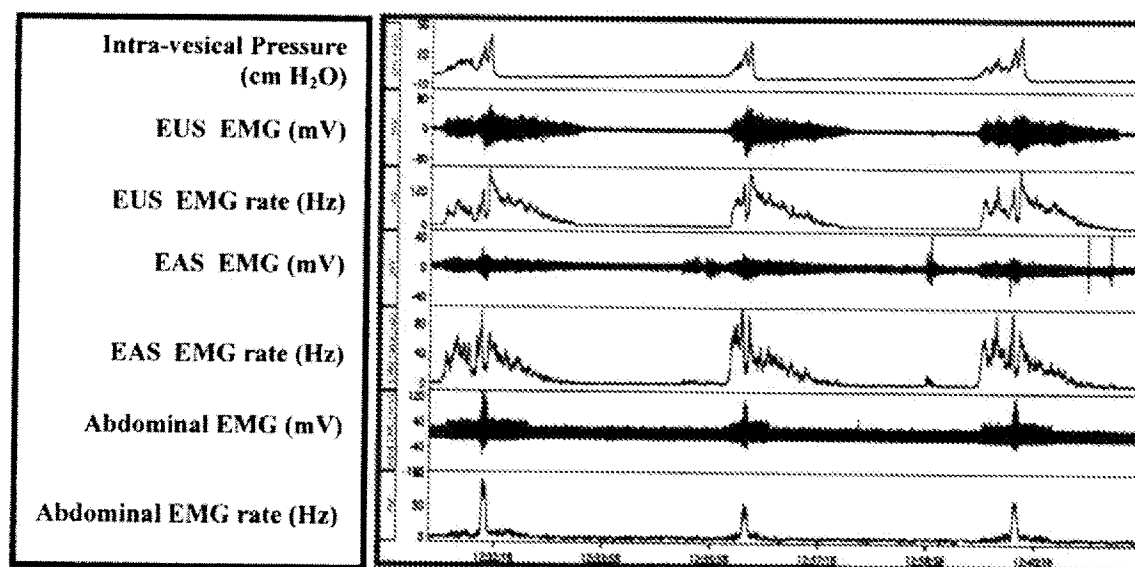
Figure 2. In vivo physiologic recording of intravesical pressure, EUS, EAS, and abdominal wall EMG during intravesical saline infusion (saline cystometrogram) (11-minute segment).

signal from the pressure transducer, which was processed using a PowerLab 8s unit data acquisition system (ADInstruments, Mountain View, CA) connected to a Macintosh G3 computer (Apple, Cupertino, CA). Cystometry catheters were calibrated with water-filled tubing attached to the transducer, the meniscus at 0 and 100 cm, relative to the height of the bladder.

Distal colon measurements. Intracolonic balloons were fashioned from condom reservoir tips and PE-50 tubing. The balloon, which approximated the dimensions of a rat stool pellet, was inserted through the anus with its proximal tip positioned 4 cm from the anal verge and was attached to the tail with adhesive tape. Via 3-way stopcocks, the intracolonic balloon was connected to a pressure transducer for measurement of intracolonic pressure and a 1-mL saline-filled syringe for balloon distention. Intracolonic pressure signals were amplified and acquired as previously described. Balloons were calibrated and zeroed outside the animal. Balloon compliance was calculated as previously described.[27]

EMG. Fine wire electrodes were fashioned from stainless steel polyurethane-coated wire (diameter, 50 μm; M.T. Giken Co, Ltd, Tokyo, Japan) and were percutaneously inserted into the external urethral sphincter (EUS), the external anal sphincter (EAS), and the lower abdominal wall musculature for EMG recording. The EMG signals were amplified using an IsoDAM8A biological amplifier (World Precision Instruments) and acquired by the PowerLab unit. The EMG signals were filtered (high frequency, 3 kHz; low frequency, 10 Hz) and acquired at a rate of 1000 samples per second. EMG frequencies were measured from the raw EMG signals using the PowerLab unit and expressed as spikes per second.

The complete in vivo experimental preparation for measuring pelvic organ (urinary bladder and distal colon) and striated musculature (EUS, EAS, and abdominal wall) reflexes and reciprocal interactions during micturition under nonirritating

and irritating conditions of either organ and/or sphincter stimulation in urethane-anesthetized rats is depicted in Figure 1. Continuous bladder filling with normal saline was initiated following surgical preparation at a rate of 0.1 mL/min; however, to ensure measurement of stable baseline pelvic organ and striated musculature activity, no control measurements were obtained until after an acclimatization period of 45–60 minutes following the acute surgical manipulations and continuous open cystometry. Following the acclimatization period, control baseline urinary bladder contraction frequency, EUS EMG activity and frequency, abdominal EMG activity and frequency, and colonic pressure and contraction parameters were then measured for 30 minutes (control period).

Colorectal distention. After completing the 30-minute control recordings, graded colorectal distentions (CRDs) were initiated while continuous bladder infusions (and micturition) continued. The intracolonic balloon was distended by graded infusions of 0.1 mL at 5-minute intervals for a total of 7 infusions (0.7 mL). Five minutes after the last infusion, the intracolonic balloon was returned to its predistention volume. Urinary bladder contraction frequency, EUS EMG activity and frequency, abdominal EMG activity and frequency, and colonic contractions were measured as previously described at each distention level. In a separate set of animals, the CRD paradigm was repeated during saline cystometry to assure stability of the response and to confirm that CRD itself did not induce colonic sensitivity.

Measurement of Bladder-to-Colon Cross-sensitization Following Bladder Irritation

Acute urinary bladder irritation was performed using intravesical infusions of protamine sulfate and potassium chloride as previously described.[28] Briefly, protamine sulfate
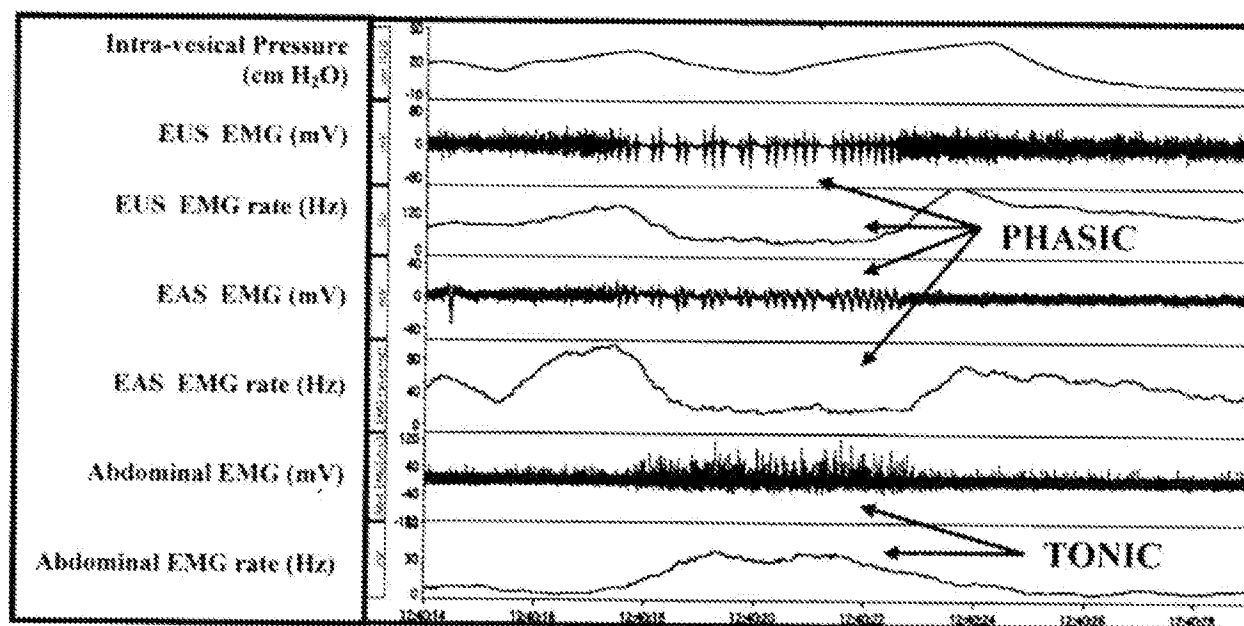
Figure 3. Urethral and anal striated sphincter activity during micturition. An expanded 15-second excerpt of the tracing in Figure 2 showing simultaneous phasic firing of the EUS and EAS and tonic firing of abdominal musculature during micturition (*arrows*).

(Sigma Chemical Co) (10 mg/mL in normal saline) was infused intravesically in place of normal saline while continuous cystometric measurements were made. Thirty minutes later, bladder infusates were replaced with 300 mmol/L KCl at the same infusion rate. Graded CRDs and cystometric recordings were then made 30–40 minutes after initiating KCl infusion, when bladder irritation was both maximal and stable. Sensory thresholds to CRD were compared before and after acute bladder irritation.

Measurement of Colon-to-Bladder Cross-sensitization Following Colon Irritation

TNBS (5% aqueous solution; Sigma Chemical Co) was instilled intrarectally as previously described by Morris et al[29] and modified by Appleyard and Wallace[30] to induce acute colonic irritation in animals undergoing saline cystometry under urethane anesthesia. Briefly, TNBS (50 mg/mL) dissolved in 50% ethanol (vol/vol) was administered via a transanal approach (total volume, 0.5 mL) using a PE-90 catheter with the tip placed approximately 4 cm proximal to the anal verge. Control animals received 0.5 mL of normal saline. Because recordings were made with rats lying in the supine position, any potential leakage of the TNBS from the colon, although not observed, would not come in contact with the perineum and hence the urethral sphincter. As an added precaution, Surgilube (E. Fougera & Co, Melville, NY) was applied to the perineum to minimize any potential contaminant irritation due to anal leakage. Following acute colonic irritation, urinary bladder contraction frequency and EUS EMG activity were then measured for 30–60 minutes.

Statistical Analysis

All data are expressed as mean ± SE and were analyzed using GraphPad Prism 3.0 statistical software (San Diego, CA). Parametric 2-way analysis of variance for repeated measures with Bonferroni's posttest was used to determine differences in CRD effects before and after acute bladder irritation. Urinary bladder intercontraction intervals (ICIs), which are indirectly related to bladder contraction frequencies, were compared in animals before and after the induction of acute cystitis or acute colitis. Paired t tests were used to identify statistically significant differences between and after each treatment. $P < .05$ was considered significant in all instances.

Results

A typical tracing illustrating rhythmic bladder activity, EUS, EAS, and abdominal wall EMG activity during the continuous infusion of saline into the bladder (saline cystometrogram) is shown in Figure 2 (11-minute excerpt). During these control conditions, the interval between urinary bladder contractions, the ICI, was 5.9 ± 0.4 minutes (n = 8) and was typical for nonirritated bladder contractions during saline cystometry at a flow rate of 0.1 mL/min in female Sprague–Dawley rats of this age.

Figure 3 represents an expanded, 15-second excerpt of the tracing in Figure 2. Note the simultaneous, phasic firing or bursting (high-frequency alternating period of EMG activity vs silence) of both the EUS and the EAS and the tonic (sustained) firing of the abdominal wall
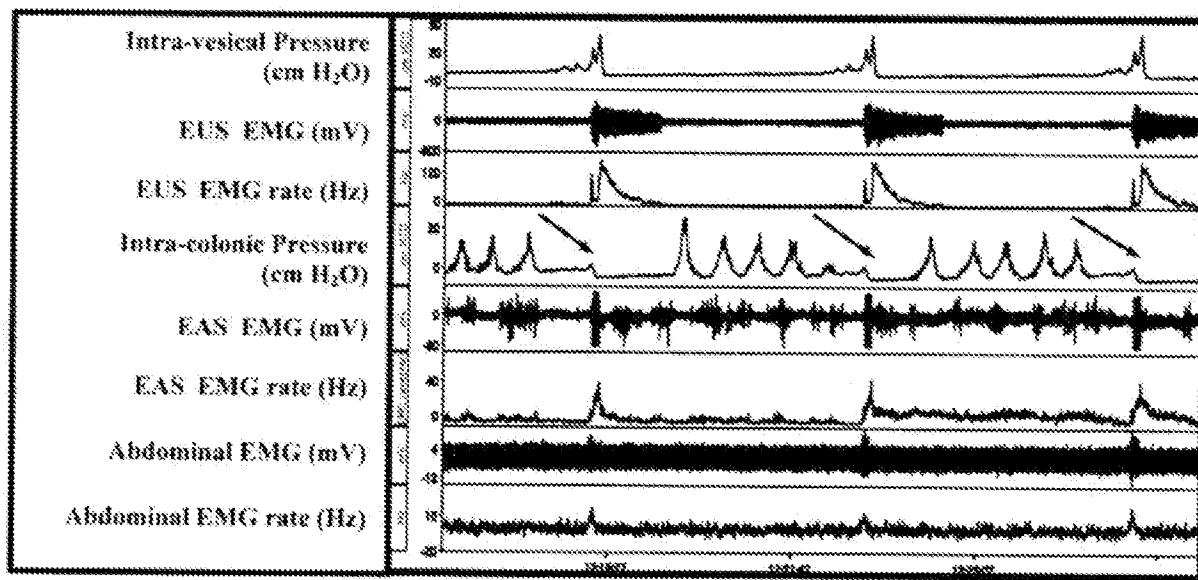
Figure 4. In vivo physiologic recording showing an inhibitory bladder-to-bowel neural reflex. Colonic activity is inhibited (*arrows*) during micturition and remains inhibited until the tonic phase of the postmicturition EUS activity subsides. Mutually independent EAS and EUS activity are also noted and include both tonic and phasic firing of the EAS independent of micturition and tonic, postmicturition EUS activity lasting 1–3 minutes following micturition-associated EUS phasic firing (14.5-minute excerpt).

during bladder emptying. During micturition, EUS firing very intimately and precisely entrained the EAS as one might expect to see in a direct neural circuit. This coordinated bursting of sphincters occurred at an average frequency of 4.3 ± 0.6 Hz. Mutually independent EAS and EUS activities were also noted and included both tonic and phasic firing of the EAS independent of micturition and tonic postmicturition EUS activity lasting 1–3 minutes following micturition-associated EUS phasic firing (Figure 4) (14.5-minute excerpt).

The measurement of distal colonic motility during saline cystometry is also shown in the tracing in Figure 4. Note the regularly paced, spontaneous colonic contractions as recorded by the intracolonic balloon. These colonic contractions occurred every 30–40 seconds when present. Interestingly, it was consistently observed that colonic activity was inhibited (Figure 4, arrows) during micturition and remained inhibited until the tonic phase of the postmicturition EUS activity subsided, suggesting a reciprocal inhibitory neural reflex (bladder-to-bowel and EUS-to-bowel reflex, respectively).

Analogously, Figure 5 shows that graded CRDs lead to inhibition of micturition and EUS activity, suggesting a reciprocal inhibitory bowel-to-bladder and bowel-to-EUS reflex. The appearance of non-micturition-associated abdominal EMG contractions, a consequence of noxious CRD, occurred after the inhibition of micturition, suggesting that the threshold for pelvic organ cross-

inhibition is lower than that for the visceromotor response to CRD. Rhythmic micturition-associated reflexes promptly returned following cessation of CRD. As expected, CRD inhibited anal sphincter activity. Cessation of CRD led to suppression of micturition-associated abdominal contractions and more prominent anal sphincter activity as compared with predistention levels. Anal distention was not associated with an increase in intravesicular pressure, but a moderate increase in EUS activity was noted (not shown) and is consistent with its role to maintain urinary continence during defecation.

The urinary bladder ICI, a measure of micturition cycle length and therefore inversely related to micturition frequency, was measured during saline cystometry and following protamine (10 mg/mL) and KCl (300 mmol/L) intravesical infusions. Protamine itself decreased the ICI by 23% compared with saline ($P < .05$), while the combination of protamine and KCl reduced the ICI by 74% ($P < .0001$) (Figure 6). The shortening of the ICI (increasing micturition frequency) reflects lower urinary tract afferent sensitization or irritation.

An example of recorded intravesical and intracolonic pressures and abdominal EMG activity during CRD in an animal before and after the induction of acute cystitis is shown in Figure 7. Graded CRDs to 60 cm H_2O produced no notable changes in abdominal wall EMG activity before the induction of acute cystitis (Figure 7A). In Figure 7B, note the decreases in the bladder ICI following intravesical irritation with protamine sulfate
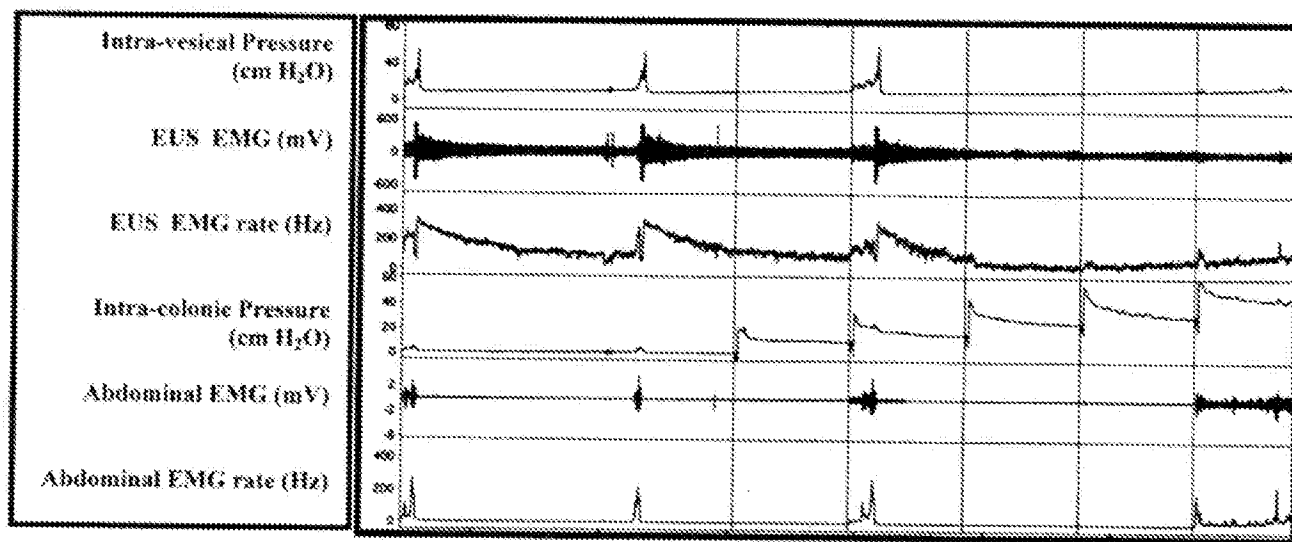
Figure 5. In vivo physiologic recording showing an inhibitory, bowel-to-bladder neural reflex. Graded CRDs lead to inhibition of micturition and EUS activity. The appearance of non–micturition-associated abdominal EMG contractions, a consequence of noxious CRD, occurred after the inhibition of micturition (24-minute tracing).

and KCl. Following acute cystitis, increases in basal and micturition-associated abdominal wall EMG activity were noted at CRD pressures much lower than 40 cm H_2O, indicating bowel hypersensitivity as a result of cross-sensitization in this model. In Figure 8, normalized abdominal wall baseline (non–micturition-associated) EMG recordings are represented for each CRD volume both before and after bladder irritation. Statistically significant differences were noted at the 0.4- and 0.5-mL CRD levels ($P < .001$). At the 0.7-mL level of CRD, the visceromotor response was equivalent in both groups.

A tracing illustrating lower urinary tract activity during continuous cystometry before and after colonic irritation with intrarectal TNBS is shown in Figure 9. Following acute colonic irritation and consistent with

Figure 6. Bladder ICIs before and after the induction of acute cystitis. *$P < .0001$.

urinary bladder sensitization, urinary bladder ICIs decreased 66% ± 3% ($P < .05$) (Figure 10). Typically, this decrease in the ICI or increase in bladder contraction frequency was noted after an immediate period of bladder inhibition lasting 2 micturition cycle lengths (ie, as early as 7 minutes).

Discussion

We have shown compelling evidence of neural cross-talk and bidirectional cross-sensitization in the pelvis using a novel experimental model. The ability to measure concurrently lower urinary tract and distal colonic sensory function and striated sphincter activity in response to cross-organ, nonirritative and irritative stimulation substantiates the importance of this model in studying pelvic pain and the overlap of CPP disorders such as IBS and IC.

As shown in Figures 4 and 5, pelvic organ, nonirritative stimulation or spontaneous physiologic activity led to apparent cross-inhibition of motor function in the nonirritated organ. Specifically, spontaneous colonic motility was inhibited during micturition, while non-noxious CRD led to inhibition of micturition. Without directly testing sensory function while eliciting these reflexes, cross-organ afferent inhibition could not be addressed in these preliminary studies, although the literature does suggest a role of a peripheral adrenergic mechanism via the hypogastric nerves[31] and a central mechanism involving pelvic nerve afferents.[31–33] Interestingly, inhibition of micturition by CRD occurred well before elicitation of the noxious visceromotor response to
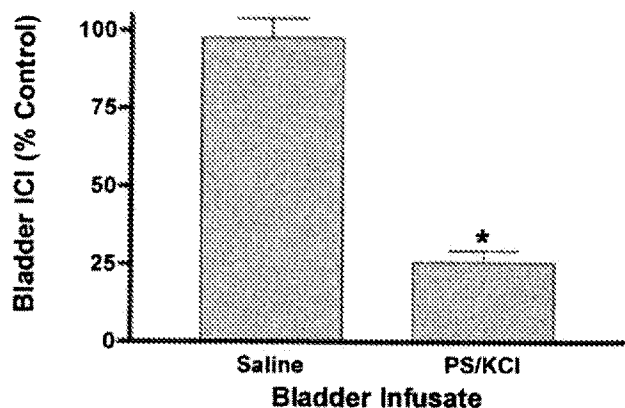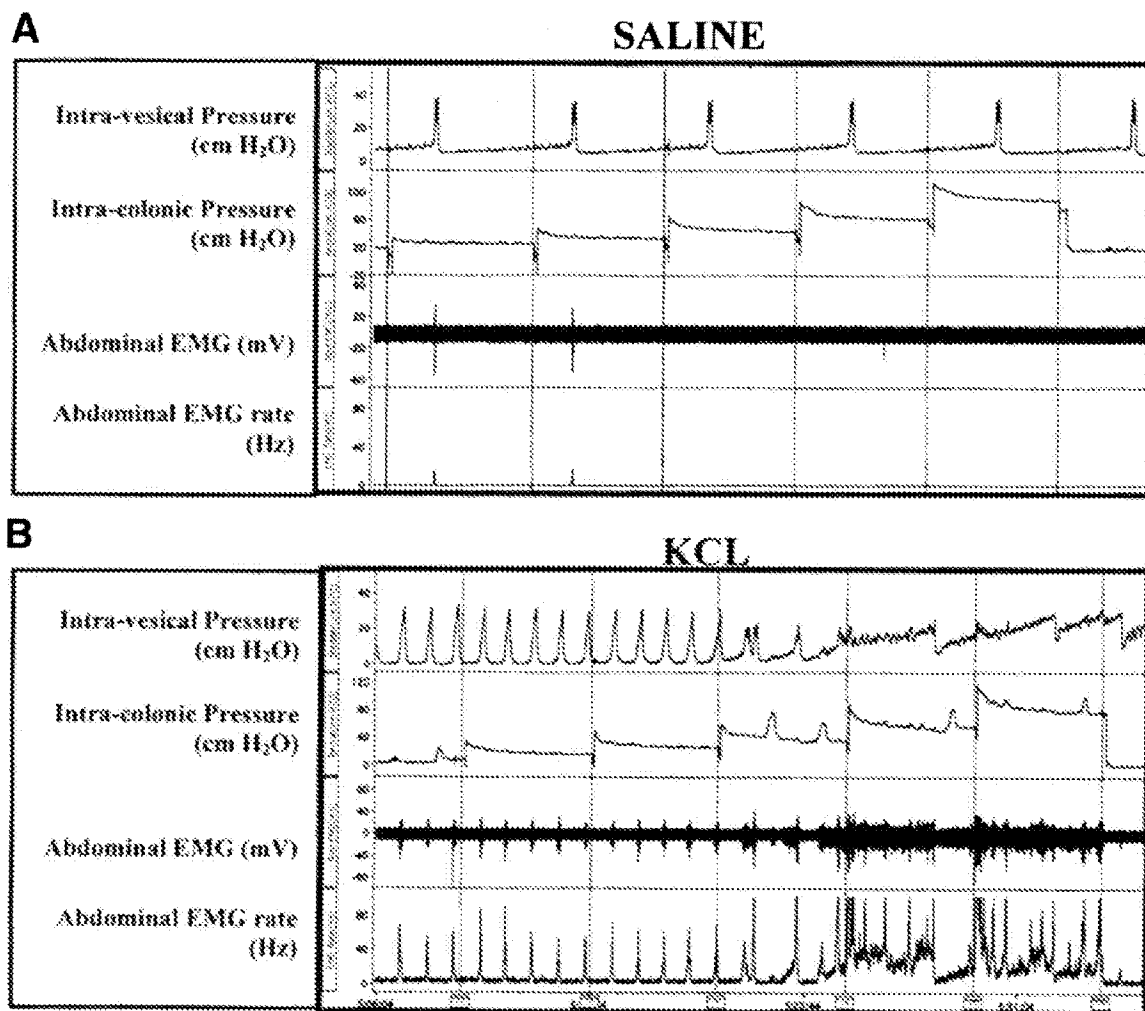
Figure 7. Sensitivity to CRD following the induction of acute KCl cystitis. (A) Non-noxious (subthreshold) CRD produced no abdominal contractions during saline cystometry. (B) In the same animal following the induction of acute cystitis, abdominal EMG responses occurred at distention levels not previously sensed (30-minute tracing).

CRD (ie, before the appearance of abdominal wall contractions) (Figure 5). This finding suggests that the bowel-to-bladder interactions and reflexes (and vice versa) involve more direct sensory pathways than those involving associated somatic afferents. Differences in sensory thresholds may also play a role. Previously, such cross-organ pelvic reflexes have been described, at least in part. Implicating bowel-bladder cross-inhibition during elimination, Denny-Brown and Robertson[34] (1933) first documented that micturition and defecation normally alternate. Furthermore, Kock and Pompeius[35] later showed (1963) that urinary bladder motility was inhibited by stimulation of the anal canal, rectum, or perineal skin. These effects were believed to be mediated by a peripheral adrenergic mechanism via the hypogastric nerves[34] and by a central mechanism involving pelvic nerve afferents as previously alluded to.[31-33] CRD pro-

duced similar results.[33] Likewise, stimulation of lower urinary tract afferents by distention of the urinary bladder inhibited distal colonic activity but increased internal anal sphincter activity.[36-38] Thus, these cross-organ pelvic reflexes elicited in this model and those described historically in the clinical literature likely represent components of a complex neural network involving sensory pathways in the pelvis that are likely important for the normal pelvic regulation and integration of sexual, bowel, and bladder function.

Figures 6 and 9 are examples of bladder-to-bowel and bowel-to-bladder (respectively) acute cross-organ sensitization. Specifically, we show that acute cystitis lowered distal colonic sensory thresholds to CRD and that acute intracolonic irritation with TNBS led to the development of a hyperactive bladder suggesting acute cystitis. Figure 8 shows the lowered thresholds to CRD in rats
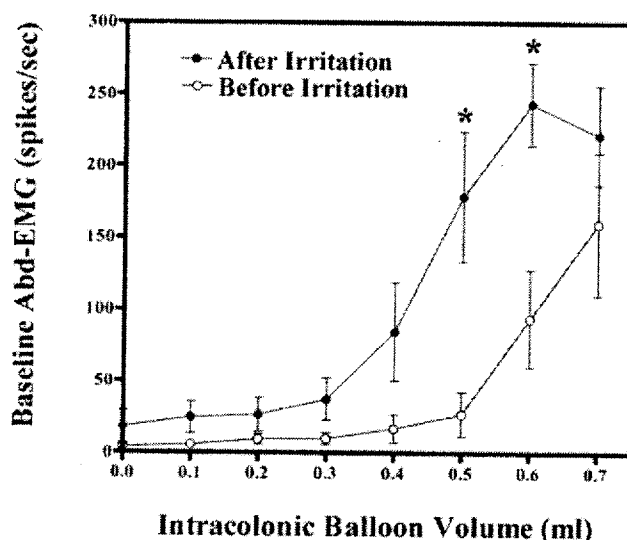
Figure 8. The effects of acute KCl cystitis on basal (non-micturition-associated) abdominal EMG activity in response to sequential intracolonic balloon distentions. *$P < .001$ (2-way repeated-measures analysis of variance; Bonferroni's posttest analysis).

exposed to acute bladder irritation but also shows that once the threshold is reached in control animals (ie, 0.7 mL), the maximal magnitude of the visceromotor response is no different than that of the irritated animals. This supports the use of abdominal EMG activity as a useful marker of visceral sensitization in our model, because organ irritation itself does not appear to exaggerate the abdominal EMG motor response in and of itself. Figure 10 illustrates a 66% decrease in the urinary bladder ICI, which is consistent with acute bladder irritation. Although urinary bladder sensitivity was not directly assessed in these studies, measurement of urinary bladder ICIs is commonly used for measuring bladder sensitivity. In the absence of overt changes in EUS EMG activity (which may affect voiding efficiency) or without application of agents that interfere with bladder contractility, this index may be used for estimation of bladder afferent sensitization. An additional, more direct measure, that of a single filling cystometry, might have been attempted, but this requires manual emptying of the bladder and interruption of the "cadence" of micturition events, both of which can alter bladder activity on their own. Therefore, as a first indication, reduction in ICI is a reasonable estimation of bladder irritation. Further studies are currently underway to take more direct measures of changes in bladder afferent sensitivity following distal colonic irritation.

The development of pelvic organ cross-sensitization in the acute setting as represented in these studies suggests a role for and subsequent modulation of preexisting afferent pathways in the pelvis. Thus, acute irritation of one pelvic organ may influence physiologic (both sensory and motor) function in another via direct neural circuits or perhaps convergent sensory input. Because neural influences on the colon, urinary bladder, and other pelvic organs are extensive, so is the potential for their dysregulation. Just as neural cross-talk in the pelvis is important for the normal regulation of sexual, bowel, and bladder function via the convergence of sensory pathways in the spinal cord,[20–25] alteration of these same convergent sensory pathways by disease or injury may play a role in the development of pelvic organ cross-sensitization, CPP, and the overlap of CPP disorders. Although Thor and Muhlhauser[59] have shown that acute chemical irritation of the urinary bladder leads to reflexive activation of motor neurons innervating the striated musculature of the external anal sphincter, experimental evidence supporting the development of cross-sensitization or afferent plasticity of one pelvic visceral organ induced by the irritation of another is limited to nonexistent. It has been previously hypothesized by Wesselmann et al[40] that a painful pelvic (possibly inflammatory) condition could develop in the referred zone of another inflamed pelvic organ via altered central mechanisms. For example, vulvar vestibulitis or prostatitis could develop in patients with IC. Historically, "referred" visceral pain as described originally by Head in 1893 represented hyperalgesia in the somatic (referred) region of a given visceral structure (viscerosomatic).[41] Possible mechanisms mediating referred visceral pain (viscerovisceral) or cross-organ sensitization in the pelvis could include (1) antidromic axon reflexes via a single primary afferent supplying 2 (dichotomizing) structures (prespinal convergence), (2) afferent-afferent interactions via spinal interneurons or overlapping terminal fields in the spinal cord (convergence-projection), (3) sympathetic reflexes, or (4) cross-sphincteric reflexes.[42]

McMahon and Morrison showed that the convergence of afferent inputs from the bowel and bladder was a common feature of visceral interneurons in the sacral cord.[26] Those interneurons identified with type II compound receptive fields were proposed as mediators of the reciprocal inhibitory reflexes between the bladder and the bowel[26] and may be an example of the convergence-projection theory proposed by Ruch.[43] Likewise, implicating spinal convergence as a mechanism of pelvic organ cross-sensitization, Qin and Foreman[44] recently showed that 32% of L6–S2 spinal neurons received convergent inputs from both the urinary bladder and the colon.

Although not well described or studied previously in the literature, viscerovisceral sensitization in the pelvis could develop by means of dichotomizing pelvic afferents whereby irritation of one pelvic organ leads to referred
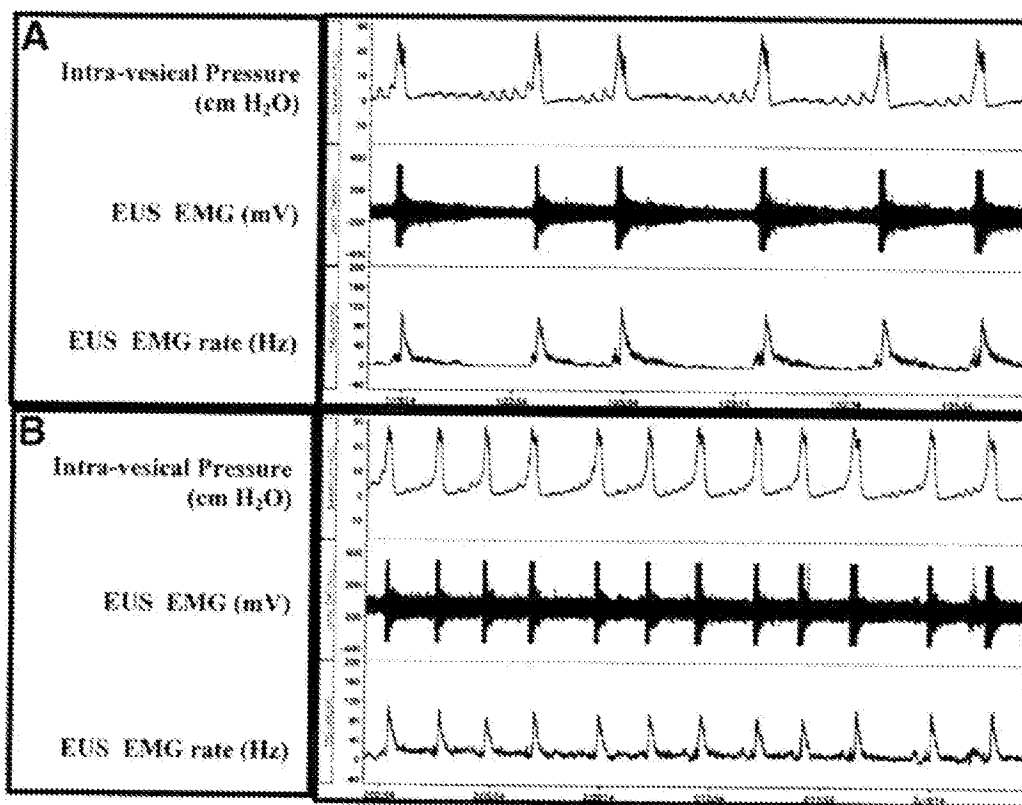
Figure 9. Lower urinary tract activity during continuous cystometry (*A*) before and (*B*) after acute TNBS-induced colitis (20-minute tracing). Note the increase in micturition frequency indicative of lower urinary tract irritation.

pain and/or neurogenic inflammation in another via afferent terminals emanating from the same dorsal root ganglion (DRG) neuron. Indeed, the presence of 2.3 times as many fibers in the dorsal root and mixed nerve as there are cell bodies in the appropriate DRG[45] is consistent with such a phenomenon. DRG neurons with dichotomizing axons were first proposed by Sinclair et al[46] and have been reported in several species and range

Figure 10. Bladder ICIs before and after intracolonic TNBS administration (n = 4). *$P < .05$.

from 0.5% to 15% of all afferents.[47] Dichotomizing afferents, which can be identified by both dual retrograde labeling studies and/or electrophysiologic recordings, have been previously identified in pelvic organs by de Groat et al.[48,49] Using retrograde tracers, de Groat et al found that 3%–6% of afferents innervating the colon and urogenital organs in both the Wistar rat and the cat were dual labeled and, thus, possibly dichotomizing. Our recent studies confirm that dual-labeled or potentially dichotomizing afferents exist in the Hilltop strain of the Sprague–Dawley rat and were as high as 15% of all bladder and colon afferents.[50] These dual-labeled DRG neurons were more predominant in the thoracolumbar populations (L1–L2), even though the majority of colonic and bladder afferents projected independently to the S1 DRG.[50] Because thoracolumbar afferents appear to be preferentially activated (normally silent) and sensitized following pelvic organ irritation,[51] it is possible that activation of these dual-target afferents may underlie the cross-organ sensitization observed in our studies described previously.

Studies in dogs showing that afferent input from the urinary bladder and the anal mucosa converge in about 90% of pontine reticular units in the pontine defecation
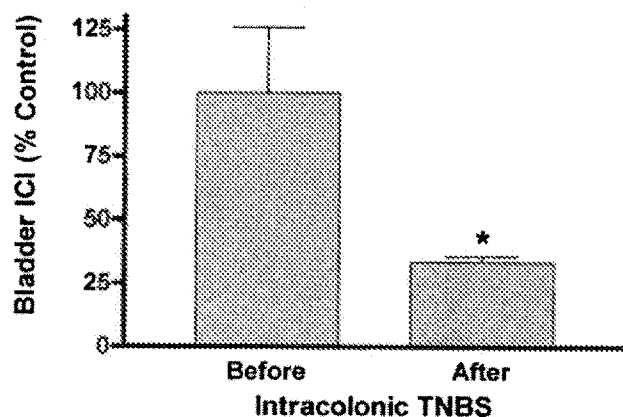
reflex center[52] are also indicative of yet another potential locus for visceral interaction involving supraspinal arcs. Likewise, 73% of bladder-responsive neurons within Barrington's nucleus in the rat were also activated by CRD.[53] In support of our findings in the pelvis, Hobson et al[54] recently found in a visceral model of foregut cross-sensitization that experimental duodenal acidification leads to the development of esophageal hypersensitivity. Central sensitization was hypothesized, although no specific mechanism was assessed. Clearly, more work needs to be performed to understand these afferent interactions in much greater detail, especially in animal models of acute and chronic pelvic inflammation.

We also report here in the rat that under conditions of normal micturition, a symphony of synchronized reflex striated muscle activity is orchestrated: phasic activity in the EUS and EAS, and tonic activity by abdominal rectus musculature. Such coordination of phasic activity of the pelvic floor musculature in the rat can easily be externally visualized under experimental conditions as pulsations of the entire female rat pelvic floor, coincident with recorded EUS (and EAS in this study) electrical activity during micturition events (M. O. Fraser, unpublished observations, March 1994). It has been shown that this coordinated activity of striated musculature contributes importantly to voiding in these rodents.[55,56] That the abdominal wall also contributes to micturition in the rat supports the notion that phasic pelvic floor activity developed in these rodents to overcome a (relatively) high-resistance urethra, because abdominal muscle contractions are generally only used in humans under conditions of outlet obstruction, such as benign prostate hyperplasia and bladder-sphincter dyssynergia, and under conditions of bladder hypocontractility.[57–59]

During micturition, EUS firing very intimately and precisely entrained the EAS, a finding one might expect to see in a direct neural circuit (Figure 3). Mutually independent EAS and EUS activities were also noted, suggesting that even though voiding-associated activity appears to be driven by a common neural oscillator for both the EUS and the EAS, the EAS also has its own neural circuitry capable of independently driving EAS phasic activity and that, likewise, EUS postmicturition tonic activity is regulated independently of the EAS.

In conclusion, these findings not only establish the feasibility of this unique model to study inflammatory disorders of the pelvis but may also enable eventual characterization of the pathophysiologic mechanisms involved in the development and overlap of IBS, IC, and other common CPP disorders. These data support the notion that the comorbidity of pelvic pain/urgency syndromes is not coincidental but rather causal in nature.

Further, neural pathways involved in the coordination of normal smooth and striated muscle activity of major pelvic organs may set the foundation for the pathophysiologic development of cross-sensitization. It may be hypothesized that long-term or ongoing stimulation of these pelvic sensory pathways and reflexes (ie, pelvic organ cross-talk) may eventually lead to more permanent sensory changes in the nonirritated organ, perhaps leading to neurogenic inflammation and sensitization via the peripheral and central release of neurotrophic factors and other mediators of this phenomenon. Clearly, further research is warranted to expand on the current findings.

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Exhibit 2:   Ustinova *et al.*, "Sensitization of the Pelvic Nerve Afferents and Mast Cell
Infiltration in the Urinary Bladder Following Chronic Colonic Irrigation is
Mediated by Neuropeptides," *Am. J. Renal Physiol.* 292:F123-130 (2007)

Sensitization of pelvic nerve afferents and mast cell infiltration in the urinary bladder following chronic colonic irritation is mediated by neuropeptides

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Ustinova EE, Gutkin DW, Pezzone MA. Sensitization of pelvic nerve afferents and mast cell infiltration in the urinary bladder following chronic colonic irritation is mediated by neuropeptides. *Am J Physiol Renal Physiol* 292: F123–F130, 2007. First published August 22, 2006; doi:10.1152/ajprenal.00162.2006.—Irritable bowel syndrome and interstitial cystitis frequently overlap. We have shown that acute colitis sensitizes urinary bladder afferents to both mechanical and chemical stimuli and that chronic colitis similarly produces neurogenic cystitis. We hypothesize that chronic irritation of the colon releases neuropeptides from bladder afferents, leading to receptor sensitization and neurogenic inflammation. Female Sprague-Dawley rats received intrarectal trinitrobenzenesulfonic acid (TNBS) or vehicle 3 days following either systemic capsaicin (CP) pretreatment or vehicle. Ten days later, action potentials of single-unit pelvic C-fiber afferents with receptive fields in the bladder were recorded under urethane anesthesia during graded bladder distensions (UBD) or intravesical capsaicin (vCP) administration. In controls, UBD increased bladder afferent firing in proportion to intravesical pressure. At intravesical pressures of 30 mmHg and above, the percent increase in afferent firing was significantly accentuated following TNBS compared with controls ($1,222 \pm 176$ vs. $624 \pm 54\%$, $P < 0.01$). The response to vCP was also enhanced ($4,126 \pm 775$ vs. $1,979 \pm 438\%$, $P < 0.01$). Systemic depletion of neuropeptides from sensory nerves abolished these effects. Histological examination of the bladders revealed an increase in mast cell density in TNBS-treated animals compared with controls (18.02 ± 1.25 vs. 3.11 ± 0.27 mast cells/ $\times 100$ field. $P < 0.01$). This effect was significantly ameliorated with CP (10.25 ± 0.95, $P < 0.5$ vs. TNBS-treated animals). In summary, chronic colonic irritation in the rat sensitizes urinary bladder afferents to noxious stimuli and causes mast cell infiltration in the bladder. Depletion of neuropeptides from sensory afferents diminishes these effects, suggesting they play an important role.

trinitrobenzenesulfonic acid; interstitial cystitis; irritable bowel syndrome; C fiber; capsaicin; mast cell

CHRONIC PELVIC PAIN (CPP) disorders such as irritable bowel syndrome (IBS), interstitial cystitis (IC), and chronic prostatitis (male CPP syndrome) affect both men and women and have a prevalence rate as high as 15% in both the United States and the United Kingdom (3, 6, 27, 30, 51). In the right setting, CPP can develop following acute or chronic irritation of individual pelvic visceral organs, their associated striated sphincters, striated muscular structures of the pelvic floor, and/or striated and cutaneous components of the pelvic abdominal wall and/or perineum (14). Because physiological activity of the colorectum and urinary bladder and their respective sensory input are a vital part of daily, conscious visceral pelvic activity (exclusive from other pelvic organs), it is not surprising that IBS and

IC, analogous disorders of pelvic visceral pain and hypersensitivity, account for one-half of all cases of CPP (50).

Although the preponderance of IBS and IC in the spectrum of CPP disorders is not entirely unexpected, their propensity to overlap and concur with other CPP disorders is quite intriguing. As many as 40–60% of patients diagnosed with IBS also exhibit symptoms and fulfill diagnostic criteria for IC, while similarly as many as 50% of patients diagnosed with IC also have symptoms and fulfill diagnostic criteria for IBS (1, 33, 36, 49). Similarly, 26% of patients diagnosed with IC were also found to have concurrent pain of the vulva or vulvodynia (17), and 45% of males with chronic prostatitis or male CPP exhibited pain with bladder filling, a classic feature of IC (30). Neural "cross-talk" within the pelvis is necessary for the normal regulation of sexual, bladder, and bowel function and is likely mediated by the convergence of sensory pathways in the spinal cord (7–11, 22).

Convergence of afferent pathways from the bladder and bowel is known to be a common feature of visceral interneurons, which are thought to mediate vesico- and colonosphincteric reflexes and colonovesical cross-inhibitory interactions (29). Fittingly, our recent studies implicated a role of preexisting neural pathways in the development of pelvic organ cross-sensitization by demonstrating that colonic hypersensitivity develops following the induction of acute cystitis and vice versa (35). Specifically, we demonstrated that acute cystitis can lower colorectal sensory thresholds to balloon distension and that acute colitis can produce acute irritative micturition patterns (35). Follow-up studies performed in our laboratory employing single unit C-fiber bladder afferent recording revealed that acute colonic irritation is capable of sensitizing urinary bladder afferents to mechanical and chemical stimuli, and interruption of the neural input to the bladder can ameliorate this effect, suggesting a direct afferent pathway from the colon (46).

Additionally, we found that chronic colonic irritation can lead to neurogenic cystitis as manifested by irritative micturition patterns, the recruitment and activation of bladder mast cells, and the upregulation of neurotrophic and mast cell growth factors in the bladder and its supplying dorsal root ganglion (DRG), which is also known to contain convergent input from the chronically irritated distal colon (Liang R, Ustinova EE, Patnam R, Fraser MO, Pezzone MA, unpublished observations). Thus, with continued irritation of a pelvic organ, neurotrophic factors produced by both smooth muscle and DRG neurons of the insulted organ (colon) may influence

neurite outgrowth and axonal sprouting at the level of the spinal cord, resulting ultimately in motor and sensory changes in other, nonirritated pelvic organs such as the bladder. Furthermore, upregulation of these same neurotrophic factors in both the nonirritated organ (bladder) and DRG containing convergent pelvic input (bowel and bladder) may account for end-organ changes such as neurogenic inflammation, afferent nerve cross-sensitivity, and axonal sprouting in the nonirritated pelvic organ (Liang R, Ustinova EE, Patnam R, Fraser MO, Pezzone MA, unpublished observations).

Thus, taking into account and expounding on our prior findings, we hypothesize that chronic irritation of the colon releases neuropeptides from bladder afferent endings leading to receptor sensitization and neurogenic inflammation. To this end, we recorded single unit C-fiber bladder activity from fine filaments of the pelvic nerve in urethane-anesthetized Sprague-Dawley female rats and assessed their responsiveness to mechanical (bladder distension) and chemical [intravesical capsaicin (vCP), bradykinin, or substance P] stimulation 10 days following intracolonic administration of trinitrobenzenesulfonic acid (TNBS) or vehicle. To evaluate the role of C-fiber afferents and their associated neuropeptides, animals were pretreated with capsaicin (CP) or vehicle 3 days before TNBS administration.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats, 200–250 g in weight, were purchased from Hilltop Lab Animals, (Scottsdale, PA) and were housed in standard polypropylene cages with ad libitum access to food (Prolab RMH 3000) and water in the University of Pittsburgh's Central Animal Facility. All studies were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee and were found to meet the standards for humane animal care and use as set by the Animal Welfare Act and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Experimental group summary. Four groups of animals were utilized in these studies. Animals were first pretreated systemically with CP or CP vehicle over a 3-day period to chronically deplete C-fiber nerve endings of their neuropeptides as previously described (21). The following day, animals were then subjected to intrarectal administration of TNBS (TNBS+) or TNBS vehicle (TNBS−) to induce colitis as previously described (35).

Electrophysiological recording of action potentials from bladder afferents traveling in the pelvic nerve and histological evaluation of the colon and bladder were performed 10 days after TNBS or TNBS vehicle administration (the chronic phase of TNBS colitis).

Depletion of C-fiber afferents. Under 4% isoflurane anesthesia, rats received on 3 consecutive days daily injections (0.2 ml sc) of CP (20, 30, and 50 mg·kg^{-1}·day^{-1}) or CP vehicle (10% ethanol and 10% Tween 80 in saline) into the fat pad on the back of the neck.

Induction of TNBS colitis. TNBS (1 M aqueous solution; Sigma) was instilled intrarectally under 4% isoflurane anesthesia as previously described by Morris et al. (31) and modified by Appleyard and Wallace (2) to induce acute colonic irritation under urethane anesthesia. Briefly, TNBS (30 mg in 50% ethanol, total volume 0.5 ml) was administered via a transanal approach using a PE-90 catheter whose tip was placed ~6 cm proximal to the anal verge. As an added precaution, Surgilube (Fougera, Melville, NY) was applied to the perineum to minimize any potential contaminant irritation due to anal leakage. This model of colitis is characterized by local areas of acute inflammation peaking at 4–7 days, followed by a chronic, mononuclear inflammatory cell infiltrate that persists up to 6 wk until it resolves without spontaneous relapse (15, 31).

In vivo physiological instrumentation. In vivo physiological instrumentation was performed as previously described (46) while the rats were under urethane anesthesia (1.2 g/kg sc, Sigma, St. Louis, MO). Following a midline laparotomy, a double-lumen transvesical catheter fashioned from PE-20 tubing (Fisher Scientific, Hanover Park, IL) was inserted through the bladder dome via a small cystotomy and ligated for urinary bladder filling and pressure recording while the bladder was maintained in its native position. One lumen of the catheter was used for introducing chemicals and draining the bladder, while the second lumen was connected to a blood pressure transducer (World Precision Instruments, Sarasota, FL) and a syringe pump (Harvard Apparatus, Holliston, MA) via three-way stopcocks for bladder filling and continuous measurement of intravesical pressure. Room-temperature saline was infused into the bladder constantly at a rate of 0.05 ml/min during continuous open cystometry. A Transbridge transducer amplifier (World Precision Instruments) was used to amplify the signal from the pressure transducer, which was processed using a PowerLab 8s unit data-acquisition system (ADInstruments, Mountain View, CA) connected to an Apple G5 computer. Cystometric catheters were calibrated with water-filled tubing attached to the transducer, the meniscus at 0 and 100 cm, relative to the height of the bladder. After a 40-min equilibration period, cystometrograms were recorded during a 30-min period, and micturition intervals were calculated for each animal.

Recording of nerve activity and identification of afferent endings. Following completion of the cystometrogram, the right pelvic nerve was isolated at the major pelvic ganglion (MPG), dissected free from surrounding tissue, and cut at a maximal distance from the ganglion. The cut end still contiguous with the bladder was positioned on a small platform and covered with mineral oil. Fine bundles were dissected and placed on one arm of a silver electrode, while a second arm was grounded. Impulses were amplified (Grass QP511; Grass, West Warwick, RI) and acquired with the PowerLab Software as above and counted in 1-s intervals. The rate meter threshold was set to count potentials of desired amplitude. A bundle that had one, or at most two, easily distinguishable active units was used. Afferent firing rate was calculated as the average number of impulses per second over a period of 20 s. Resting activity represented maximal activity recorded 1 min before each intervention. Changes in afferent activity in response to interventions were expressed as percent change from the resting firing rate.

Only spontaneously active afferents that had precise receptive fields in the bladder (i.e., afferents clearly responding to probing of the bladder surface with a fine-tipped rod) were studied. Conduction velocity was estimated by measuring the distance between the receptive field and recording electrode and dividing it by the latency between electrical stimulation of the receptive field and evoked potential. Afferent recording was limited to unmyelinated C fibers as characterized by conduction velocities <2.5 m/s and capsaicin sensitivity (38). Fibers with high conduction velocities and not responsive to CP were discarded.

Mechanical and chemical testing of afferents. After identification of the sensory ending, the mechanical sensitivity of the afferent was tested by distension of the bladder (UBD) with saline infusion at the rate of 0.25 ml/min to the maximal intravesical pressure of 60 mmHg. During the infusion, the external urethral sphincter was clamped to maintain pressure in the bladder. Afterward, the bladder was immediately emptied and returned to a baseline pressure of 4–6 mmHg. UBDs were repeated two to three times within 10- to 15-min intervals to ensure the stability of the response. Afferent firing during bladder distension was averaged over 10-mmHg increments of intravesical pressure.

Chemical sensitivity of the afferent was tested with CP (0.1–10 μg in 0.2-ml total volume; 0.1 ml of capsaicin solution followed by 0.1-ml saline flush). The response of the afferent to CP was compared with the response to administration 0.2 ml of saline. Responses to CP vehicle (10% ethanol in saline) were tested and found to be no

different from responses to the same volume of saline. Due to the differences in baseline firing rate in the individual afferents, changes in afferent activity were also expressed as percent change from baseline.

Some afferents were also tested with intravesical administration of bradykinin (1–100 μg) or substance P (1–100 μg). All chemicals were purchased from Sigma. Responses of the afferents to chemical stimulation were measured as the average number of impulses per second over a period of 20 s during 1 min following chemical administration. All chemicals were instilled in a total volume of 0.2 ml, which predictably increased intravesical pressure by 10–12 mmHg in all cases.

Although the urothelium is thought to be an impermeable barrier to intravesical agents, drugs with high lipophilicity such as CP easily penetrate the urothelium and consequently exert their affects on C-fiber afferents. Less lipophilic drugs less easily penetrate the urothelium, but absorption still occurs. From our own experience, the required dose of such drugs needed to produce a response is at least 10 times higher than if applied on the serosal surface of the bladder.

Morphology and mast cell histology. Four animals from each experimental group were euthanized using pentobarbital sodium (100 mg/kg ip, Abbott Laboratories, North Chicago, IL). The distal colon was dissected from the anus to the splenic flexure, longitudinally opened, and examined macroscopically. Representative cross-sectional colonic tissue samples located 6 cm from the anus (i.e., the site of TNBS administration) were embedded in OCT medium (Miles Laboratories, Elkhart, IN) and frozen. The urinary bladders were excised and dissected longitudinally along their midline. Both halves were embedded in OCT medium and frozen. Using a cryostat (Microm HM 505 E, Mikron Instruments, San Marcos, CA), multiple sections from both organs were obtained at a thickness of 5 μm. Serial sections were mounted and stained with hematoxylin/eosin and Giemsa stains (25). Mast cells were counted independently by two investigators under a ×100-power field as previously described (39). Ten to twenty fields were counted for colon and bladder sections from each animal, and the average number of mast cells per field was calculated.

Statistical analysis. Reported values represent means ± SE. Data were analyzed using GraphPad Prism 3.0 statistical software (San Diego, CA). Differences between groups were determined by ANOVA, and differences between means were isolated by a Bonferroni correction for multiple *t*-tests. Statistical significance was accepted at $P < 0.05$.

RESULTS

Effects of CP pretreatment and TNBS-induced colitis on micturition rates. As shown in Fig. 1 and corresponding to a 42% increase in micturition rate, voiding intervals were significantly decreased 10 days following intrarectal TNBS treatment (CP−/TNBS+). The average micturition interval was 307 ± 28 s in control animals (CP−/TNBS−) vs. 177 ± 21 s in TNBS-treated animals (CP−/TNBS+) ($P < 0.01$). CP pretreatment alone (CP+/TNBS−) had no effect on voiding frequency; however, CP pretreatment completely abolished the decrease in the voiding interval induced by TNBS (CP+/TNBS+). There were no cystometric abnormalities noted during the filling phases of the micturition curves to suggest detrusor overactivity in the TNBS-treated animals.

Effects of CP pretreatment and TNBS-induced colitis on bladder afferent responses to UBD. Action potentials were recorded from 26 single-unit bladder afferents in 15 control rats (CP−/TNBS−) and from 18 single-unit bladder afferents fibers in 9 rats with TNBS-induced colitis (CP−/TNBS+). No significant differences in the afferent resting firing rates were
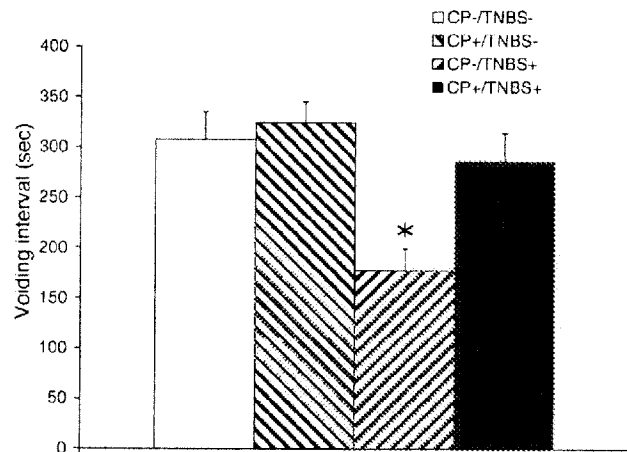

Fig. 1. Effects of capsaicin (CP) pretreatment and trinitrobenzenesulfonic acid (TNBS)-induced colitis on micturition rates. Voiding intervals during saline cystometry are represented for CP−/TNBS−, CP−/TNBS+, CP+/TNBS−, and CP+/TNBS+ treatment groups. Voiding intervals were significantly decreased by 42% following intrarectal TNBS treatment (CP−/TNBS+) (*$P < 0.01$). CP pretreatment alone (CP+/TNBS−) had no effect on voiding frequency; however, CP pretreatment completely abolished the decrease in the voiding interval induced by TNBS (CP+/TNBS+).

noted between these two groups (0.44 ± 0.08 impulses/s for controls and 0.57 ± 0.19 impulses/s following TNBS). Similarly, neither CP pretreatment alone (CP+/TNBS−; 12 afferents from 7 rats) nor CP pretreatment followed by TNBS administration (CP+/TNBS+; 15 afferents from 8 rats) changed the resting firing rate of bladder afferents (i.e., 10 days post-TNBS or -TNBS vehicle administration; 0.37 ± 0.17 impulses/s for CP+/TNBS− vs. 0.46 ± 0.19 impulses/s for CP+/TNBS+). Bladder afferent responses to UBD with saline are summarized in Fig. 2 for all four groups. As seen in Fig. 2 in all four groups, UBD increased bladder afferent activity in proportion to intravesical pressure. This distension-induced enhancement in bladder afferent firing was more pronounced in the TNBS-treated rats (CP−/TNBS+) as evidenced by the greater slope (Fig. 2). At intravesical pressures of 10–20 mm (nonnoxious range), bladder afferent activity in all groups was equivalent; however, at UBD pressures of 30 mmHg and above, bladder afferent activity was substantially increased in the CP−/TNBS+ group (1,222 ± 176 vs. 624 ± 54% in CP−/TNBS− at 30 mmHg, $P < 0.05$).

CP pretreatment (CP+/TNBS−) had no significant effects on the afferent responses to UBD compared with controls. In the group of animals that received TNBS after pretreatment with capsaicin (CP+/TNBS+), the responses of the afferents to UBD were significantly attenuated compared with the TNBS alone group (CP−/TNBS+) and equivalent to controls (CP−/TNBS− and CP+/TNBS−).

Effects of CP pretreatment and TNBS-induced colitis on bladder afferent responses to chemical irritation with vCP. Figure 3 illustrates bladder afferent responses to intravesical infusion of saline and vCP compared with baseline activity. Increases in bladder afferent activity in response to intravesical saline infusion (0.2 ml; corresponding to low intravesical pressure) were no different across experimental groups including those receiving TNBS. Although animals pretreated with CP-pretreated tended to have less of an increase in afferent
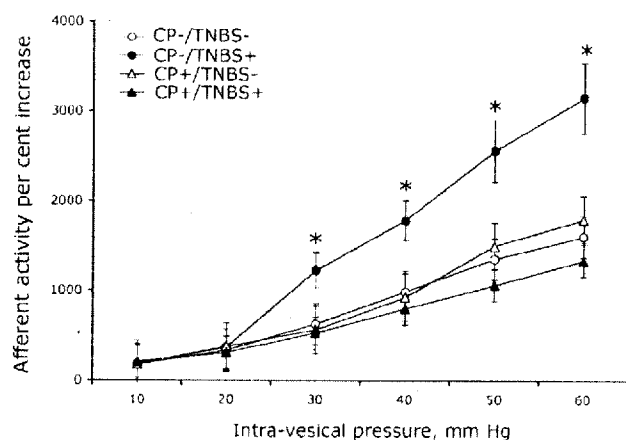
Fig. 2. Effects of CP pretreatment and TNBS-induced colitis on bladder afferent responses to bladder distensions (UBD). Percent increases in bladder afferent activity during UBD from 10 to 60 mmHg are represented for CP−/TNBS−, CP−/TNBS+, CP+/TNBS−, and CP+/TNBS+ treatment groups. Ten days following intracolonic TNBS treatment (CP−/TNBS+), bladder afferent responses were significantly increased beginning at 30 mmHg. This effect was completely blocked with CP (CP+/TNBS+), which alone had no effect on bladder afferent responses ($*P < 0.05$ vs. CP−/TNBS−).

firing in response to saline infusion, this trend was not significant.

In response to vCP, the bladder afferent firing rate was markedly increased in the CP−/TNBS+ group (4,126 ± 775 vs. 1,979 ± 438% for CP−/TNBS−, $P < 0.01$). Pretreatment with CP alone (CP+/TNBS−) had no statistically significant effect on the responses of the afferents to vCP compared with controls (CP−/TNBS−), although the increase in afferent responses to vCP in animals with TNBS-induced colitis was abolished (1,450 ± 357% in CP+/TNBS+ vs. 4,126 ± 775% in CP−/TNBS+ $P < 0.01$).

Effects of CP pretreatment and TNBS-induced colitis on bladder afferent responses to chemical irritation with intravesical administration of bradykinin and substance P. Fifteen bladder afferents from the control group (CP−/TNBS−) were

Fig. 3. Effects of CP pretreatment and TNBS-induced colitis on bladder afferent responses to chemical irritation with intravesical administration of CP. Bladder afferent responses to intravesical saline infusion (0.2 ml) 10 days following TNBS administration were no different from controls. Although CP-pretreated animals tended to have smaller increases in bladder afferent activity, this trend was not statistically different. Bladder afferent responses to intravesical CP infusion (0.2 ml) were significantly increased in TNBS-treated animals (CP−/TNBS+) ($*P < 0.01$ vs. CP−/TNBS−). This effect was blocked by CP pretreatment (CP+/TNBS+).
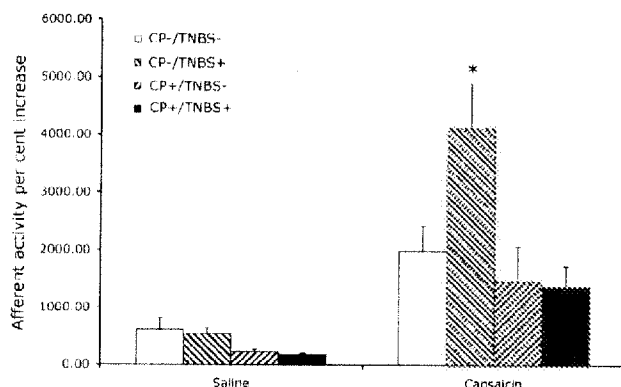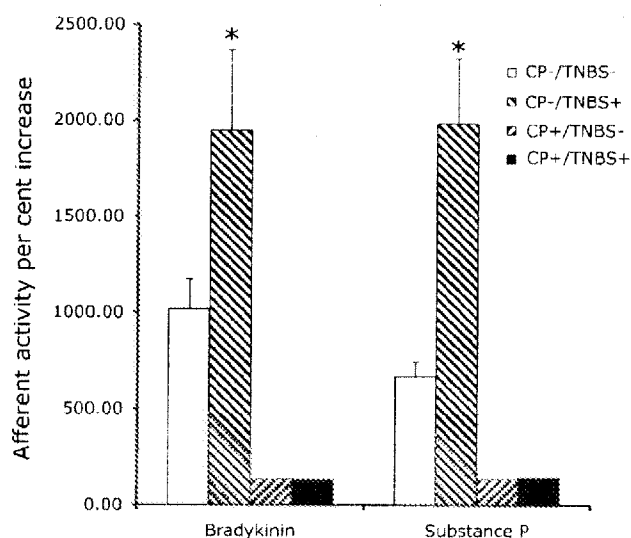

Fig. 4. Effects of CP pretreatment and TNBS-induced colitis on bladder afferent responses to chemical irritation with intravesical administration of bradykinin and substance P. In rats pretreated with TNBS, bladder afferent responses to chemical irritation with bradykinin (10 μg) and substance P (10 μg) were substantially enhanced. All effects were blocked completely by capsaicin pretreatment ($*P < 0.05$ vs. CP−/TNBS−).

further assessed for bradykinin and substance P responsiveness. Eight of the 15 bladder afferents tested from the control group responded to intravesical administration of bradykinin (1–100 μg), and 12 of 15 were found to be activated with substance P (1–100 μg). Intrarectal administration of TNBS (CP−/TNBS+) did not significantly change the proportion of afferents responding to either of the chemicals: 12 of 19 tested afferents responded to bradykinin, and 14 of 15 were activated with substance P. TNBS, however, did dramatically increase the magnitude of the responses to both chemicals (Fig. 4). Specifically, the response of the afferents to bradykinin increased from 1,013 ± 159 to 1,813 ± 419 ($P < 0.05$), while the response to substance P increased from 665 ± 75 to 1,987 ± 338% ($P < 0.05$).

Pretreatment with CP completely abolished the responses of bladder afferents to intravesical administration of bradykinin and substance P in both CP pretreatment groups (CP+/TNBS− and CP+/TNBS+).

Morphology and mast cell counts in distal colon and urinary bladder. In the colon, none of the animals in control (CP−/TNBS−) or CP pretreatment (CP+/TNBS−) groups showed macroscopic abnormalities (Fig. 5). In contrast, animals in both groups that received TNBS (CP−/TNBS+ and CP+/TNBS+) showed marked macroscopic alterations in the distal colon (5–7 cm from the anus). These alterations included mucosal edema, bowel wall thickening, erosions, and ulcerations. All of these changes were more pronounced in the CP+/TNBS+ group and, in three of four rats, were accompanied by an incomplete bowel obstruction with proximal colonic dilatation. There were no cases of gross bowel perforation.

Microscopically, distal colonic sections from control and CP-pretreated groups showed normal a colonic wall with focal mild submucosal edema. Occasional mast cells were present (0.8 ± 0.3 and 1.5 ± 0.5 cells/×100-field in CP−/TNBS− and CP+/TNBS− groups, respectively, $P = 0.124$, not significant) (Fig. 6).
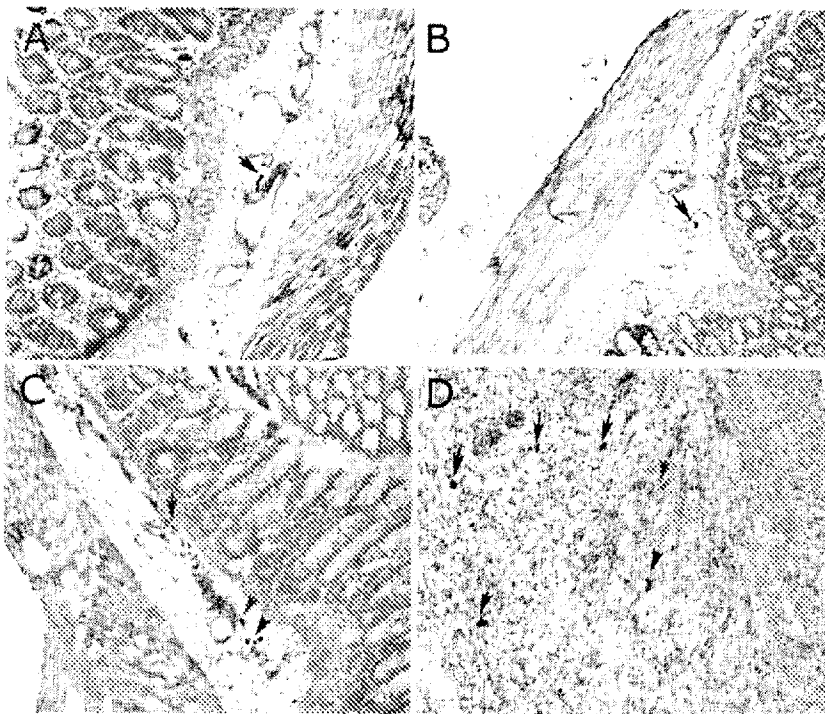
Fig. 5. Distal colon histological sections stained with Giemsa. Representative images were taken from CP−/TNBS−−, CP+/TNBS−−, CP−/TNBS+−, and CP+/TNBS+-treated animals (A–D, respectively). The densely staining granular cells (arrowheads) represent mast cells (×100 power).

Microscopic changes in the colons of the animals treated with TNBS (CP−/TNBS+ and CP+/TNBS+ groups) consisted of severe mucosal damage, characterized by focal ulcerations with associated polymorphonuclear and histiocytic infiltrates. The mucosa adjacent to ulcers revealed edema, hemorrhage, and distortion of crypt architecture. Mast cell infiltration was present in the lamina propria and muscularis propria (Fig. 5). The number of mast cells per ×100-power field in CP−/TNBS+ and CP+/TNBS+ groups was significantly higher than in the control group (3.0 ± 1.1 and 5.9 ± 1.0, respectively, vs. 0.8 ± 0.3, $P < 0.05$) (Fig. 6).
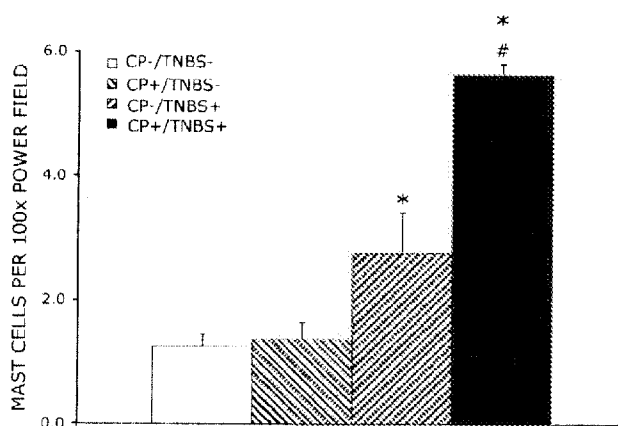

Fig. 6. Distal colon mast cell quantitation. Mast cells were quantitated in CP−/TNBS−, CP+/TNBS−, CP−/TNBS+, and CP+/TNBS+ treatment groups under ×100 magnification. Colonic mast cell numbers were significantly elevated in CP−/TNBS+ and CP+/TNBS+ treatment groups compared with controls. The number of mast cells in the CP+/TNBS+ group was significantly higher (2-fold) than those of the CP−/TNBS+ group. *$P < 0.05$ vs. CP−/TNBS−. #$P < 0.05$ vs. vs. CP−/TNBS+.

Gross examination of the urinary bladders did not reveal macroscopic abnormalities in any of the experimental groups. Microscopically, in CP−/TNBS− and CP+/TNBS− groups, bladder morphology was normal, whereas in CP−/TNBS+ and CP+/TNBS+ groups there was mild to moderate submucosal edema. No epithelial damage or polymorphonuclear infiltrate was seen. Mast cells were predominantly located around submucosal and adventitial blood vessels with occasional infiltration of the lamina propria and muscularis propria (Fig. 7). The principal microscopic alteration was an increase in the number of mast cells in the bladder in the animals with TNBS-induced colitis (Fig. 8). In TNBS-treated animals, the number of mast cells per ×100-power field was significantly higher (18.02 ± 1.25 and 10.25 ± 0.95 in CP−/TNBS+ and CP+/TNBS+ groups, respectively) than in groups not treated with TNBS (3.11 ± 0.27 and 5.4 ± 0.50 cells in CP−/TNBS− and CP+/TNBS− groups, respectively) (Fig. 8).

Contrary to the mast cell counts in the colon, the number of mast calls in the bladder was significantly higher in the CP−/TNBS+ group than in the CP+/TNBS− group: 18.02 ± 1.25 vs. 10.25 ± 0.95, $P = 0.036$ (Fig. 8).

DISCUSSION

Corroborating our acute studies of pelvic organ cross-sensitization (35, 46), our current findings provide compelling evidence that chronic colonic irritation (10 days post-intrarectal TNBS administration) directly sensitizes the mechano- and chemoreceptive properties of urinary bladder C fibers traveling within the pelvic nerve. In our original studies, cross-organ pelvic reflexes and acute cross-organ irritative alterations in physiological functioning and sensation (bladder-to-bowel and vice versa) were described, and the development of cross-sensitization in this setting suggested a role for, and subsequent

Fig. 7. Urinary bladder histological sections stained with Giemsa. Representative images were taken from CP−/TNBS− (A), CP+/TNBS− (B), CP−/TNBS+ (C), and CP+/TNBS+ treatment groups (D). The densely staining granular cells (arrowheads) represent mast cells. (×100 power).
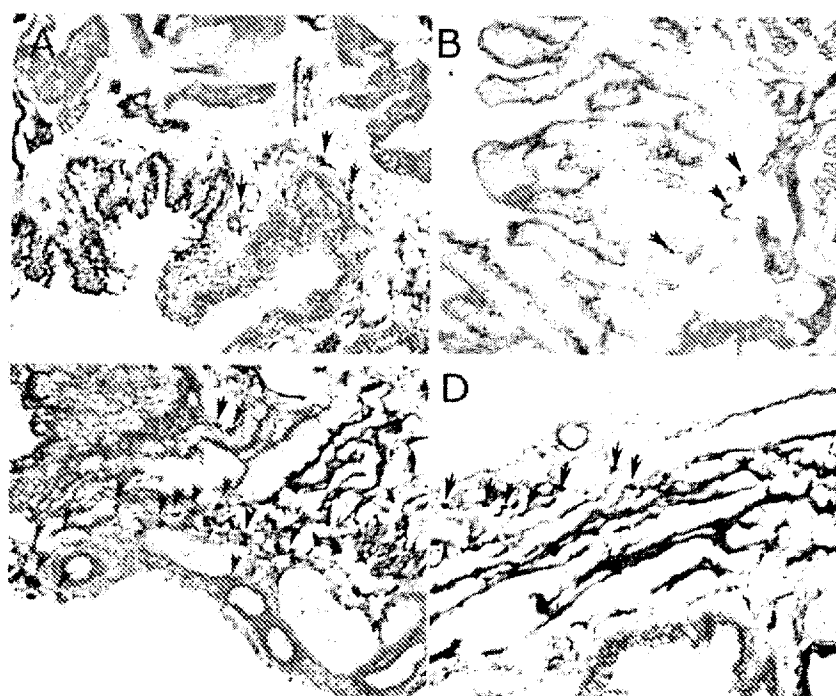
modulation of, preexisting afferent pathways in the pelvis (35). This role of preexisting afferent pathways was further supported in our acute studies (1 h post-TNBS administration), where we first showed that colonic irritation is capable of sensitizing urinary bladder afferents to mechanical and chemical stimuli, a mechanism found to be directly dependent on neural input to the bladder (46). Correspondingly, in the chronic setting, by showing that CP pretreatment completely ameliorates these effects (both for mechanical and chemical urinary bladder stimulation), a role of C-fiber afferents and the release of their active neuropeptides in this pelvic cross-sensitization process are further substantiated. Such afferent cross-sensitization pathways (both acute and chronic) may
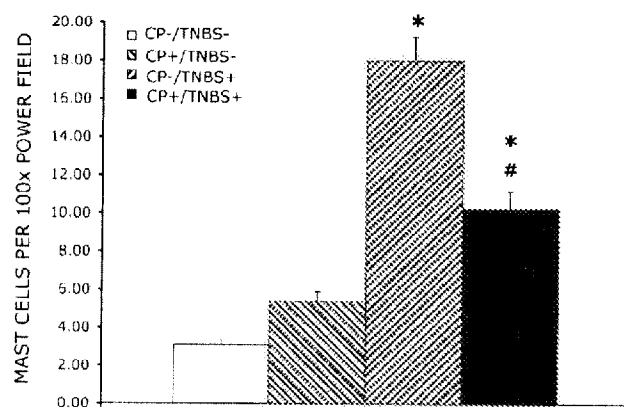

Fig. 8. Urinary bladder mast cell quantitation. Mast cells were quantitated in CP−/TNBS−, CP+/TNBS−, CP−/TNBS+, and CP+/TNBS+ treatment groups under ×100 magnification. Urinary bladder mast cell numbers were significantly elevated in CP−/TNBS+ and CP+/TNBS+ treatment groups compared with controls. The number of mast cells in the CP−/TNBS+ group was significantly higher (almost 2-fold) than in the CP+/TNBS+ treatment group. $*P < 0.05$ vs. CP−/TNBS−. $\#P < 0.05$ vs. CP−/TNBS+.

originate centrally via spinal or supraspinal circuits (including spinal antidromic dorsal root reflexes) and/or peripherally, directly from the colon via antidromic axon reflexes from a single dichotomizing primary afferent supplying two structures (prespinal convergence) (5).

Although bladder C-fiber sensitization in response to intrarectal TNBS was noted in both our acute (46) and our current chronic studies, unlike in the acute setting, basal bladder afferent firing 10 days after intrarectal TNBS administration had completely returned to control levels (equivalent to TNBS vehicle). The normalization of basal bladder afferent activity during this 10-day interval adds further credence to this model of chronic bladder hyperalgesia and provides further experimental support for the postinfectious or postirritative disease models of IBS, IC, and other CPP disorders (40, 47), where often an initial pelvic organ insult leads to visceral hypersensitivity that only becomes apparent when physiological stimuli exceed the diminished visceral afferent sensory thresholds. Similarly, in our chronic studies the associated development of neurogenic cystitis as evidenced by increased mast cell counts in the absence of macroscopic bladder damage is also supportive of our disease hypothesis as it also reflects human correlational studies for IBS, IC, and other pain syndromes (34, 37, 43, 45, 48).

As shown in Fig. 2, at intravesical pressures of 10–20 mm (nonnoxious range), bladder afferent activity in all groups was equivalent; however, at UBD pressures of 30 mmHg and above, bladder afferent activity was substantially increased in the animals treated with TNBS alone (CP−/TNBS+). Because bladder C-fiber afferents typically respond to "noxious" intravesical pressures (30–50 mmHg) under normal conditions (20), it is not unexpected that their greatest increase in magnitude following cross-organ sensitization would occur in that same pressure range as we have shown previously (46).

Evidence for chemical sensitization of bladder afferents was not limited to intravesical CP irritation. Responses to bradykinin and substance P were similarly accentuated 10 days following TNBS. These findings support those in the literature showing that chemically induced cystitis in animals is associated with sensitization of chemosensitive afferents and/or recruitment of afferents normally unresponsive to mechanical stimulation (12, 13, 20). Inflammatory mediators, such as prostaglandin E_2, serotonin, histamine, and adenosine, as well as nerve growth factor, can modulate the functional properties of C-fiber afferents, leading to their hyperactivity and increased excitability (4, 12, 16, 19), and these changes in C-fiber afferent properties likely translate into increased pain sensation (18, 20). The modulation of these responses with systemic CP pretreatment further substantiates the role of C-fiber afferents in our model of pelvic organ bladder cross-sensitization. Although such afferent cross-sensitization pathways may involve central and/or peripheral afferent circuits (5) and although systemic CP does not preferentially affect individual pelvic organs, a direct role of bladder afferent endings was previously substantiated in our acute studies in which selective bladder denervation completely ameliorated these cross-sensitization effects (46). Moreover, amelioration of the effects of TNBS by bladder denervation confirmed that local diffusion or systemic effects of the intracolonic irritant did not account for our findings (46).

As expected, marked macroscopic and microscopic alterations including mastocytosis were noted in the distal colon 10 days following intrarectal TNBS administration. All of these changes were more pronounced in the CP+/TNBS+ treatment group, suggesting a protective role of CP-sensitive afferents in colonic mucosal injury, a phenomenon described previously in both the fore- (42) and hindgut (28). Gross examination of the urinary bladder, however, did not reveal macroscopic abnormalities in any of the experimental groups. Furthermore, microscopically, no epithelial damage or polymorphonuclear infiltrate was seen at 10 days, and this lack of bladder injury is in agreement with our acute studies of colonic irritation (46) as well as those of others (26). Interestingly, as noted in the colon (although at the site of direct mucosal injury), the number of bladder mast cells was significantly increased following TNBS-induced colitis (Fig. 7). In contrast to the colon, bladder mast cells were predominantly located around submucosal and adventitial blood vessels, with occasional infiltration of the lamina propria and muscularis propria, suggesting a neurohumoral pattern of chemotaxis.

The observed urinary bladder mastocytosis in this setting is not unexpected as a contributing role of mast cells in IC (45) and IBS (34, 37, 48) and in other disorders characterized by hyperalgesia and neurogenic inflammation has been previously implicated (43). There is a preponderance of evidence implicating potential pathophysiological mast cell-nerve interactions in the sensitization of visceral afferent nerves as the mast cell possesses a formidable armamentarium of nociceptive molecules including adenosine phosphates, bradykinin, histamine, leukotrienes, potassium, lymphokines, tumor necrosis factor (TNF), and prostaglandins (44). Anatomic evidence further supporting this mast cell-mediated modulation of afferent nerve function is apparent in the close apposition of mast cells to nerve fibers in both the human gastrointestinal tract (32, 41) and the urinary bladder (23).

Further supporting a neurogenic pathogenesis of the observed bladder mastocytosis, we previously demonstrated increased urinary bladder expression of stem cell factor and nerve growth factor, two potent mast cell growth and sensitizing factors following both acute and chronic colonic irritation with TNBS (Liang R, Ustinova EE, Patnam R, Fraser MO, unpublished observations). Furthermore, the differential effects of CP pretreatment on bladder and colonic mastocytosis in response to intrarectal TNBS likely also reflect differing mechanisms of mast cell stimulation. Accordingly, the near normalization of bladder mast cells in animals pretreated with CP before TNBS is consistent with a neurogenic mechanism of stimulation, while in the colon, the direct tissue insult and impairment of protective mechanisms following CP pretreatment were sufficient stimuli to enhance the mast cell response (42).

In summary, chronic colonic irritation in the rat with TNBS sensitizes urinary bladder afferents to mechanical and chemical stimuli and induces bladder mastocytosis. A role of C-fiber afferents was further substantiated in this model of neurogenic cystitis as CP pretreatment significantly ameliorated these effects. Thus these data provide further support for neural processes in mediating cross-sensitization of pelvic organs and the overlap of IC, IBS, and other CPP disorders.

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Exhibit 3: Sarna, "Enteric Descending and Afferent Neural Signaling Stimulated by Giant Migrating Contractions: Essential Contributing Factors to Visceral Pain," *Am. J. Physiol. Gastrointest. Liver Physiol* 292:G572-581 (2007)

Enteric descending and afferent neural signaling stimulated by giant migrating contractions: essential contributing factors to visceral pain

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Enteric Neuromuscular Disorders and Visceral Pain Center, Division of Gastroenterology, Departments of Internal Medicine, Neuroscience and Cell Biology, The University of Texas Medical Branch at Galveston, Galveston, Texas

Sarna SK. Enteric descending and afferent neural signaling stimulated by giant migrating contractions: essential contributing factors to visceral pain. *Am J Physiol Gastrointest Liver Physiol* 292: G572–G581, 2007. First published September 21, 2006; doi:10.1152/ajpgi.00332.2006.—We investigated whether strong compression of an intestinal segment by giant migrating contractions (GMCs) initiates pseudoaffective signals from the gut, similar to those initiated by its distension with a balloon. The experiments were performed on conscious dogs by using close intra-arterial infusions of test substances that affect the receptors only in the infused segment. The stimulation of GMCs by close intra-arterial infusion of CGRP or distension of an intestinal segment by balloon increased the heart rate; the increase in heart rate was greater when the balloon distension and GMCs occurred concurrently in separate intestinal segments. The suppression of contractility in the distended segment blocked the increase in heart rate. By contrast, the stimulation of rhythmic phasic contractions (RPCs) or their spontaneous occurrence did not increase the heart rate. The occurrence of GMCs as well as intestinal distension also produced descending inhibition. The descending inhibition was blocked by the inhibition of nitric oxide synthase, but it was unaffected by the inhibition of adenylyl cyclase, purinergic receptors P2X and P2Y, and muscarinic receptors M_1 and M_2. The synaptic transmission for descending inhibition was mediated primarily by nicotinic receptors and activation of nitric oxide synthase. It was unaffected by the inhibition of tachykinin receptors NK_1, NK_2, and NK_3; serotonin receptors $5\text{-}HT_{1A}$, $5\text{-}HT_2/5\text{-}HT_{1C}$, $5\text{-}HT_3$, and $5\text{-}HT_4$; and muscarinic receptors. Our findings show that GMCs, but not RPCs, initiate pseudoaffective signals from the gut. In the presence of visceral hypersensitivity or impaired descending inhibition, the GMCs may become a noxious stimulus.

irritable bowel syndrome; inflammatory bowel disease; peristaltic reflex; nitric oxide; vasoactive intestinal peptide; high-amplitude propagating contractions; constipation; functional bowel disorders

INTERMITTENT ABDOMINAL CRAMPING of gut origin is one of the major symptoms in patients with irritable bowel syndrome (IBS) (13, 14) and inflammatory bowel disease (IBD) (2, 39). Visceral hypersensitivity has been identified as a contributing factor to this symptom (22, 28, 50, 67). However, the sensation of cramping in these patients is intermittent. Therefore, stable visceral hypersensitivity alone could not explain the intermittent occurrence of abdominal cramping. The sensation of cramping in these patients and in normal subjects is mimicked by intraluminal balloon distention beyond nociceptive threshold. This suggests that a mechanical stimulus from the gut wall is an essential requirement for the perception of intermittent pain of gut origin. This stimulus is likely to be the contractions of the gut wall. However, there is little information on which type or types of contractions (29, 57) may stimulate afferent signaling that is perceived to be painful by the central nervous system (CNS) or trigger pseudoaffective responses, such as increase in heart rate and abdominal wall contractions (52). The measurement of pseudo-affective reflexes is used routinely in animal studies as proxy for afferent signaling that may be perceived as painful in humans. Our first hypothesis is that giant migrating contractions (GMCs) (13, 14, 17, 21, 36, 43, 45, 54, 56) but not the rhythmic phasic contractions (RPCs) (57) stimulate afferent signaling that initiates pseudoaffective responses.

GMCs of the small intestine are large-amplitude (2–3 times greater than the maximum amplitude of RPCs during phase III activity of the migrating motor complex) and long-duration (4–6 times longer than the duration of an RPC) contractions (56). As a result, these contractions strongly occlude a 20- to 30-cm-long segment of the small intestine. Furthermore, these contractions propagate rapidly and uninterruptedly over long distances. The luminal contents trapped ahead of a GMC are, therefore, propelled rapidly over long distances (mass movements) (17, 45, 63). The large volume of luminal contents propelled rapidly by a GMC may distend the distal receiving segment. A GMC, therefore, may stimulate afferent signaling directly by strong compression of the intestinal wall and indirectly by distention of the distal receiving segment. The distention of the distal segment can be mimicked by inflation of an intraluminal balloon. Our second hypothesis is that the GMCs, but not RPCs, initiate the descending inhibition that allows the distal receiving segment to distend without stimulating afferent signaling and hence nociceptive perception. The impairment of the descending inhibition, however, may initiate afferent signaling concurrent with that produced by the GMCs so that the two afferent signals add up to exceed the nociceptive threshold, even in the absence of visceral hypersensitivity.

The descending inhibition in the gut is comprised of two components: *1*) synaptic transmission via interneurons so that the inhibition can extend over a sizable length of the gut ahead of a GMC and *2*) radial or lateral input to circular smooth muscle cells by enteric inhibitory motor neurons so that it can overcome the existing excitatory effects of the cholinergic excitatory motor neurons. Extensive in vitro studies have identified the neurotransmitters and receptor subtypes that mediate these two components of descending inhibition (9, 19, 32, 33, 42, 46). However, this information is not available in the intact conscious state. We, therefore, investigated the roles

of key neurotransmitters and receptor subtypes involved in synaptic transmission in the interneurons and inhibition of smooth muscle contractions by the inhibitory motor neurons to produce descending inhibition in intact awake dogs. The antagonists were infused into the intestinal wall by close intra-arterial infusions in doses that are established to be selective in blocking their enteric targets but have no systemic effects (30, 40, 55, 58, 59, 68).

EXPERIMENTAL METHODS

Surgical procedure. The experiments were performed on 10 healthy conscious dogs of either sex. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the Zablocki Veterans Affairs Medical Center, Milwaukee, WI. Access to the abdominal cavity was obtained under general pentobarbital sodium anesthesia (30 mg/kg iv; Abbott Laboratories) to surgically attach strain-gauge transducers to the seromuscular layer to record circular muscle contractions and implant Silastic catheters in the ileal mesenteric arteries to infuse test substances directly into short segments of the ileum.

Three main adjacent mesenteric arteries labeled as proximal, middle, and distal, ~30 to 40 cm apart, were identified in the ileum. The arteries were freed carefully from the mesentery, preserving the nerves. A Silastic catheter (0.75 mm internal diameter, 1.63 mm external diameter) was inserted in the centripetal direction in a branch artery of each of the three main arteries so that its tip rested 1 to 2 mm from the junction of the branch artery and the main artery, as described previously (30, 40, 58). The infusion of saline at 15 to 20 ml/min for 10 to 15 s identified the boundaries of the infused segment. The segment refilled with blood within 2 to 3 s after the end of infusion. The infusion of saline at 1 ml/min for up to 10 min produced no apparent change in color of the segment and it did not stimulate any contractions. The length of the infused segment was limited to ~6 cm by ligating some secondary branch arteries, if necessary. Ties to the branch artery and the mesentery secured the catheters. Two or three strain-gauge transducers were attached to the seromuscular layer in each of the proximal (*catheter 1*), middle (*catheter 2*), and distal (*catheter 3*) infused segments to record circular muscle contractions.

A stainless steel cannula was implanted ~10 to 15 cm proximal to the site of the proximal catheter and one the same distance distal to the site of the distal catheter. A 6-cm-long balloon connected to a catheter was advanced through the proximal cannula to determine the position that placed the balloon at the site of the proximal infused segment. This mark was used later in experiments to position the balloon at this location to distend the proximal segment. The distal balloon was used for concurrent distension of its segment and stimulation of GMCs in the proximal segment. These two sites were far apart to interfere with each other.

The intraluminal and intra-arterial catheters were exteriorized subcutaneously in the subscapular region. The catheters were housed in jackets that the dogs wore at all times. Each intra-arterial catheter was flushed twice daily with 2,000 IU of heparin. The dogs were allowed 5–7 days to recover from surgery.

Experimental protocol. All experiments were performed in the conscious state after an overnight fast. At least one phase

III activity was recorded to establish the fasting state. The contractile signals were recorded on a 12-channel pen recorder (model 7D; Grass Instruments, Quincy, MA), with lower and upper cutoff frequencies set at direct current and 15 Hz, respectively.

All test substances were infused through the catheters at 1 ml/min during phase I or a quiescent period during phase II activity of the migrating motor complex. Close intra-arterial infusion of calcitonin gene-related peptide (CGRP) through the proximal catheter was used to stimulate GMCs, as described previously (58). The balloon was used to distend an intestinal segment, while measuring the pressure in the balloon with a gauge. Close intra-arterial infusions of methacholine (MCh) through the distal catheter were used to stimulate a series of RPCs. The antagonists of specific receptors were infused through the middle and distal catheters as described in RESULTS.

A waiting period of at least 30 min was allowed between successive infusions of agonists and antagonists. Preliminary experiments indicated that the responses to repeated infusions of agonists after this rest period were not different.

Data analysis. GMCs defined as contractions of duration four to six times longer and amplitudes two to three times larger than those of contractions in phase III activity of the migrating motor complexes were identified visually (56). The RPCs were quantified as area under contractions (WINDAQ/EX program; DATAQ Instruments, Akron, OH). The area under contractions was measured from the beginning of the first contraction after the start of infusion to the point at which the tracing returned to baseline and contractions ceased to occur.

All data are expressed as means $\pm$ SE. The n value represents the number of dogs. Statistical analysis was performed by analysis of variance with repeated measures. Student-Newman-Keuls test was used for multiple comparisons when the data were distributed normally, whereas Mann-Whitney's rank sum test was used when the normality test failed; $P < 0.05$ was considered statistically significant.

RESULTS

Pseudoaffective responses to balloon distension and GMCs. We used the increase in heart rate to monitor the pseudo-affective response to distension of an intestinal segment with a balloon and its strong compression by a GMC. The distention of the proximal segment with a balloon for 5 min increased the heart rate in proportion to the distention pressure (Figs. 1A and 2A). The maximal increase in heart rate was achieved within the first minute of balloon distention and it was sustained during the entire 5-min distention period. The close intra-arterial infusions of 2 μM/min $\times$ 1 min CGRP stimulated one to four GMCs, some of which propagated distally. This dose of CGRP was shown earlier to consistently stimulate GMCs in the canine small intestine (58). The maximal increase in heart rate measured over 1-min during the occurrence of GMCs (135 $\pm$ 3%) was not significantly different from that attained during balloon distention to 290 mmHg (140 $\pm$ 6%) (Fig. 2B). However, the maximum increase in heart rate produced by the GMCs occurred only after one or two GMCs had begun to propagate. The maximal increase in heart rate when an intestinal segment distal to *catheter 3* was distended to 290 mmHg concurrently with the stimulation of GMCs by CGRP in the
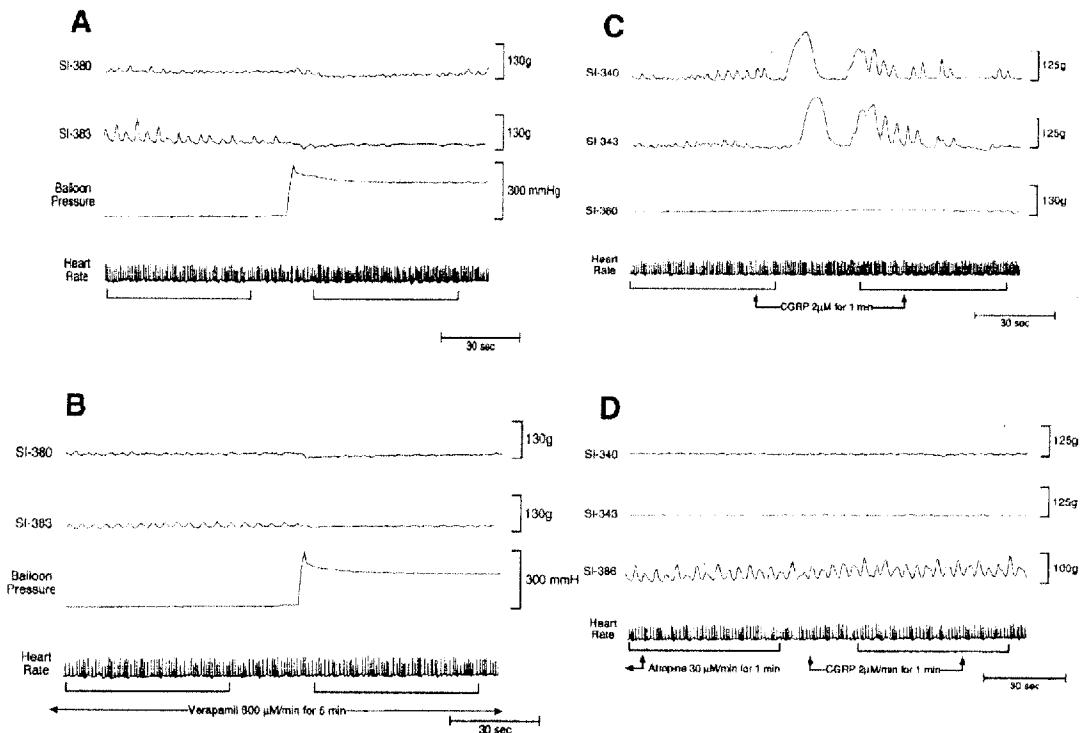
A

B

C

D

Fig. 1. *A*: the distension of a balloon at the site of proximal catheter increased the heart rate within a few seconds. *B*: the balloon was distended at the site of the proximal catheter 3 min after the start of infusion of verapamil through this catheter. The suppression of contractility of the intestinal segment blocked the increase in heart rate by balloon distension. *C*: the close intra-arterial infusion of CGRP stimulated giant migrating contractions (GMCs) at the site of proximal catheter and the GMCs propagated distally. The strong compression of the intestinal segment by the GMCs also increased the heart rate. *D*: the stimulation of GMCs by CGRP was blocked by a prior close intra-arterial infusion of atropine through the same catheter. The inhibition of GMCs blocked the increase in heart rate seen in *C*. SI, small intestinal strain gauge transducer. Numbers after SI indicate the distances of the transducers from the pylorus (in cm).

proximal infused segment (159 ± 4%) was significantly greater than that produced by each stimulus separately (Fig. 2*B*). The infusion of 1 ml/min × 5 min 0.9% saline had no significant effect on heart rate (100 ± 0 before vs. 92 ± 4 during saline infusion, $n = 5$)

We then investigated whether the increase in heart rate was due to strong compression of the intestinal segment by the GMCs or to the direct action of CGRP on its enteric neural receptors. The stimulation of GMCs by CGRP infusion was blocked either by a 1-min prior close intra-arterial infusion of 30 μM/min × 1 min atropine or by starting the CGRP infusion during the third minute of infusion of 800 μM/min × 5 min infusion of verapamil, an L-type Ca^{2+} channel blocker. Both antagonists blocked the stimulation of GMCs by CGRP, as reported previously (58), and they also blocked the increase in heart rate (Figs. 1*D* and 3). Both agents also blocked the increase in heart rate produced by distention of the proximal segment with the balloon (Figs. 1*C* and 3). The balloon was distended 2 min after the start of infusion of verapamil. These data suggested that the suppression of contractility in an intestinal segment prevents afferent signaling from it in response to its distension or strong compression. Close intra-arterial infusions of atropine and verapamil alone had no significant effect on heart rate (100 ± 0 vs. 97 ± 3 after atropine infusion, and 100 ± 0 vs. 97 ± 5 during the fifth minute of verapamil infusion, $n = 7$ and 4, respectively). The control heart rates were measured within 5 min prior to the stimulus.

By contrast, close intra-arterial infusions of 2 μM/min × 5 min MCh stimulated a series of RPCs that had no significant effect on the heart rate (100 ± 0 vs. 95 ± 10 after 1-min infusion of MCh $n = 5$). Similarly, the heart rate did not vary with different intensities and frequencies of rhythmic phasic contractions during phases I, II, and III of the migrating motor complex (MMC) cycle in the ileum (100 ± 0, 96 ± 6 and 94 ± 5 in phases I, II and III, respectively, $n = 5$).

Neurotransmitters and receptors that mediate descending inhibition by balloon distention and GMCs. The following experiments investigated *1)* the roles of putative neurotransmitters and receptors associated with the inhibitory motor neurons and smooth muscle cells that mediate descending inhibition and *2)* the roles of selective neurotransmitters and receptors that mediate synaptic transmission during descending inhibition. They also investigated whether balloon distention that is normally used as an experimental stimulus and GMCs that occur spontaneously in the intact conscious state (45, 56) use the same or different neural signaling pathways to produce descending inhibition.

Balloon distention to 290 mmHg or stimulation of GMCs by close intra-arterial infusion of CGRP in the proximal segment was employed as stimulus to produce descending inhibition. The descending inhibition by balloon distension was measured by determining its effect on a series of RPCs stimulated at the site of the distal catheter by close intra-arterial infusion of 2 μM/min MCh for 1 min (Fig. 4*A*). MCh was infused during the third minute of balloon distention (Fig. 4*B*). Initial experiments
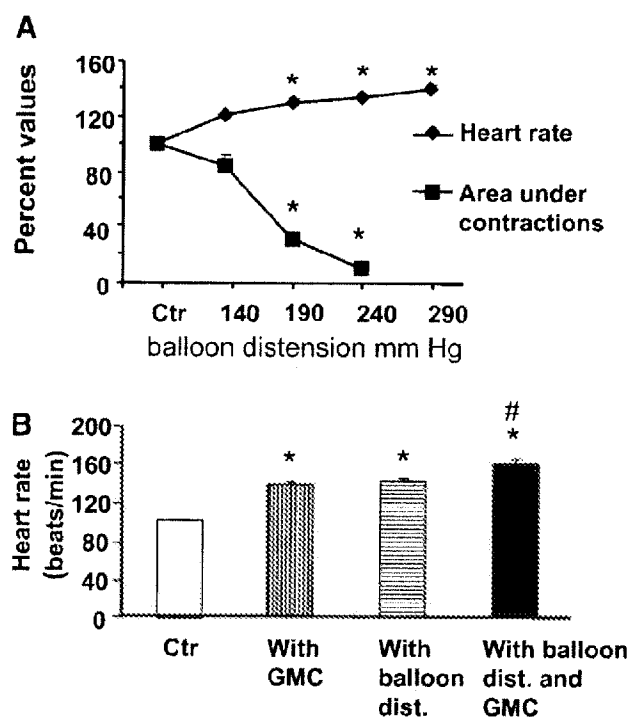
A

B

Fig. 2. *A*: balloon distension (dist.) increased the heart rate proportional to distension pressure. The balloon distension also inhibited methacholine (MCh)-induced contractions at the site of distal catheter. The descending inhibition was also proportional to distension pressure. Ctr, control. *B*: the increases in heart rates with distension of intestinal segment by balloon and its compression by GMCs were of the same order of magnitude. However, the increase in heart rate by concurrent distension and compression of different segments was greater than that by each stimulus alone. *$P < 0.05$ with respect to control, #$P \leq 0.05$ with respect to GMC and balloon distension, $n = 5$.

indicated that descending inhibition produced by balloon distention was almost immediate and it was sustained during the entire period of distention. The area under RPCs stimulated by MCh without balloon distention was taken as 100%.

For GMC experiments, the RPCs were stimulated by a 5-min infusion of MCh at the site of the distal catheter (Fig. 5*A*). The descending inhibition produced by GMCs was not immediate upon their onset. The GMCs propagated distally at a velocity of 0.29 ± 0.02 cm/s (Fig. 5*B*). The first GMC had to propagate to a distance of 37 ± 4 cm before the MCh-induced contractions began to be inhibited. At this time, the GMCs were ~30 cm from the site of the distal catheter. The CGRP infusion at the proximal catheter and MCh infusion at the distal catheter were begun at the same time (Fig. 5*C*). The area under the contractions during the fifth minute of infusion of MCh and without the concurrent infusion of CGRP was taken as 100%. The inhibitory effect of propagating GMCs on MCh-induced RPCs always began before the start of the fifth minute of MCh infusion.

Balloon distention as well as stimulation of GMCs at the site of the proximal catheter significantly inhibited the contractions stimulated by MCh at the site of the distal *catheter 3* (Figs. 4*B*, 5*B*, and 6, respectively). The inhibition of nitric oxide (NO) synthase (NOS) at the site of *catheter 3* by 10 mM/min × 1 min infusion of *N*-nitro-L-arginine methyl ester (L-NAME) given 12-min prior to the infusion of MCh blocked the balloon

and GMC-induced descending inhibitions (Figs. 4*C*, 5*D*, and 6). However, the inhibition of adenylyl cyclase by infusion of 1 μM/min × 5 min MDL-1233A; M_1 receptors by infusion of 2 μM/min × 5 min pirenzepine; M_2 receptors by 2 μM/min × 5 min infusion of methoctramine; P2X receptors by 2 μM/min × 5 min infusion of PPADS; P2Y receptors by infusion of 200 μM/min × 5 min infusion of suramine or 100 μM/min × 5 min infusion of reactive blue (data not shown) did not block descending inhibition by balloon or GMCs (Fig. 6). The depletion of norepinephrine by intravenous administration of 7.5 μM/min × 1 min of guanethidine also had no significant effect on descending inhibition produced by balloon distention or by GMCs. The effective concentrations of close intra-arterial infusions of antagonists were determined by their ability to block the inhibition of RPCs by their respective agonists (Fig. 7) or from the literature (30, 38, 40, 55, 59, 68).

The roles of specific neurotransmitters and their receptors involved in synaptic transmission for descending inhibition were determined by distending the balloon in the proximal segment, stimulating RPCs in the distal segment, and blocking selective receptors in the middle segment by close intra-arterial infusions. The inhibition of nicotinic receptors by infusion of 70 mM/min × 1 min hexamethonium or of NOS by 10 mM/min × 1 min infusion of L-NAME in the middle segment blocked the descending inhibition produced by balloon distention (Fig. 8*A*). On the other hand, the infusion of 30 μM/min × 1 min atropine or 1 ml/min × 1 min of 0.9% saline (not shown) in the middle segment had no effect on descending inhibition produced by balloon distention (Fig. 8*A*).

Furthermore, the blockade of NK_1, NK_2, and NK_3 receptors by 1.6 μM/min × 5 min infusions of L-703.606, L-659.877, and [Trp^7, β, Ala^8] neurokinin A_{4-10}, respectively, and of 5-HT_{1A}, 5-HT_2/5-HT_{1C}, 5-HT_3/5-HT_4 receptors by 2 μM/min × 5 min infusions of NAN-190 HBr$_2$, LY-53857, tropesitron, and SDZ-205557, respectively also had no significant effect on descending inhibition by balloon distention (Fig. 8, *B* and *C*).

DISCUSSION

Our findings show that the distention of an intestinal segment by an intraluminal balloon or its strong compression by GMCs stimulates centrally detectable afferent signals (pseu-
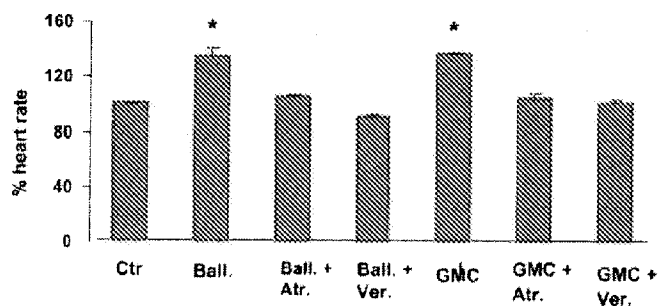

Fig. 3. Balloon (Ball.) distension and stimulation of GMCs both increased heart rate. However, when the contractility of the distended segment or the segment in which CGRP was infused to stimulate GMCs was suppressed by prior close intra-arterial infusions of atropine (Atr.) or verapamil (Ver.), the increase in heart rate was blocked. *$P < 0.05$ with respect to control, $n = 5$ or 6.
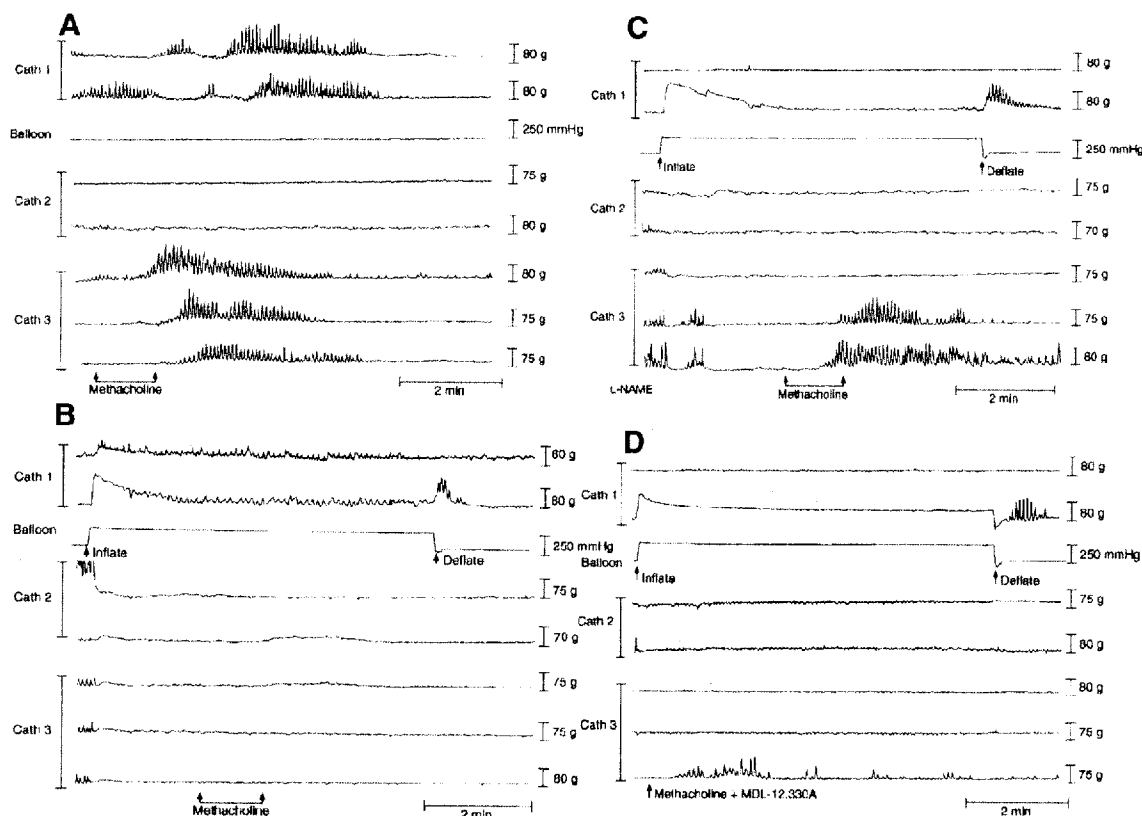
Fig. 4. One-minute infusion of MCh stimulated a series of rhythmic phasic contractions (RPCs) at the site of the distal catheter (Cath). The area under these contractions at one of the strain-gauge transducers was taken as 100%. *B*: balloon distension to 290 mmHg at the site of the proximal catheter inhibited these contractions completely. *C*: the inhibition of nitric oxide synthase (NOS) by a prior close intra-arterial infusion of *N*-nitro-L-arginine methyl ester (L-NAME) at the site of the distal catheter blocked the descending inhibition of MCh-induced contractions by balloon distension at the proximal site. *D*: however, the inhibition of adenylyl cyclase to block the action of VIP had no effect on descending inhibition by balloon distension.

do-affective responses) that increase the heart rate. The increase in heart rate stimulated by intestinal distention or its strong compression by GMCs is of the same order of magnitude. However, the increase in heart rate by compression of an intestinal segment by GMCs and concurrent distension of another intestinal segment by balloon is greater than that due to each stimulus alone. In animal models of visceral pain, the initiation of pseudo-affective responses, such as abdominal wall contractions and increase in heart rate, have been used as markers of stimuli that can be perceived as painful (52). Our findings show, therefore, that the strong compression of a gut segment by a GMC can induce the sensation of pain. By contrast, the RPCs of maximal amplitude and frequency during phase III activity of the migrating motor complex may not be able to induce the sensation of pain.

Intermittent visceral pain of gut origin is one of the major symptoms of IBS (26, 45, 70, 67), IBD, and gut inflammation (2, 12, 25, 44, 47, 49, 66, 67). The initial hypothesis was that this pain was due to motility dysfunction (60, 65), but there was little evidence to support this hypothesis. The occurrence of GMCs as distinct contractions was not widely known at that time, and the focus was on RPCs. More recently, the motility hypothesis has been discounted and the alternate hypothesis that the symptom of intermittent pain is almost entirely due to hypersensitivity of afferent sensory neurons and/or overinter-

pretation of these signals in the CNS has been advanced (10, 16, 22, 50). It has been proposed also that the motility dysfunction in IBS results from afferent neural hypersensitivity (10), but there is no evidence to support it. Although hypersensitivity of the afferent neurons is well established in a subset of IBS patients (1, 18, 70), it alone does not explain the intermittent occurrence of abdominal cramping. In a clinical laboratory setting, the patients complain of abdominal pain only when a balloon is distended in their gut lumen above the nociceptive threshold, even though hypersensitivity is continually present. This suggests that a mechanical event in the gut is required to stimulate afferent signals to perceive pain of gut origin. Our findings show that this pathophysiological mechanical event is the GMC. The afferent signaling stimulated by GMCs is similar to that stimulated by balloon distention. The RPCs are almost always present somewhere in the gut. If these contractors were the stimulus for pain, the pain would be present at all times. Furthermore, our findings show that RPCs do not generate centrally detectable afferent signals.

The GMCs occur spontaneously in normal healthy subjects two to six times per day (3, 6, 13, 43, 48, 51, 56, 57, 62). These spontaneous GMCs in health occur primarily in the terminal ileum and in the proximal colon; in the distal colon they also precede defecation (3, 37, 43, 48). In all cases, they produce mass movements (17, 45, 63), but they are seldom perceived to
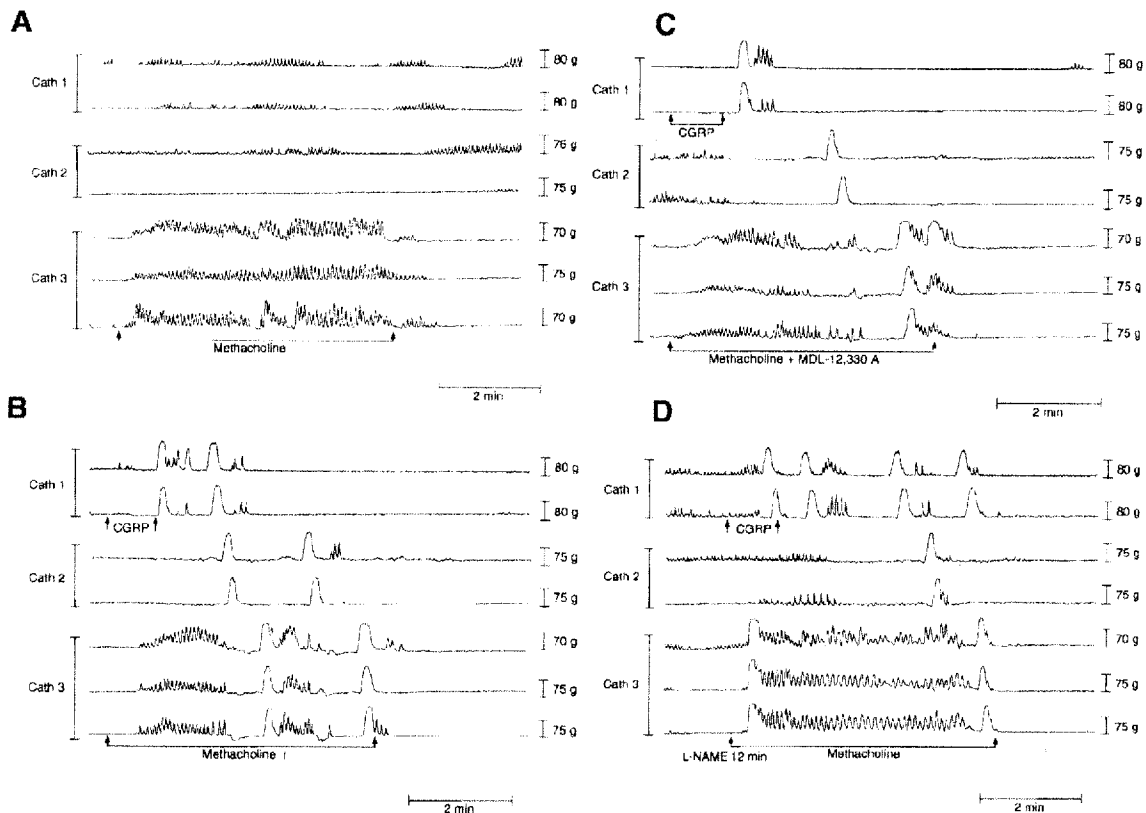
Fig. 5. *A*: a 5-min infusion of MCh stimulated a series of RPCs at the site of the distal catheter. The area under these contractions at one of the strain-gauge transducers during the 5th minute of infusion was taken as 100% *B*: a close intra-arterial infusion of CGRP at the proximal catheter stimulated 2 GMCs that propagated distally. The MCh-stimulated RPCs at the site of the distal catheter were inhibited when the propagating GMCs reached ~30 cm from the site of RPCs. *C*: the inhibition of adenylyl cyclase by MDL-12330 A did not block descending inhibition ahead of distally propagating GMCs. *D*: the blockade of NOS by L-NAME almost completely blocked descending inhibition ahead of distally propagating GMCs.

be painful, even though they stimulate afferent signaling as shown by our findings. The reason seems to be that the afferent signals stimulated by the GMCs are still subthreshold for nociception, even though they exceed the affective threshold (Fig. 9). The afferent signaling by GMCs may be perceived as painful *1*) if their occurrence is accompanied with hypersensitivity of the afferent neurons and/or overinterpretation of the afferent signals in the CNS so that the nociception threshold is

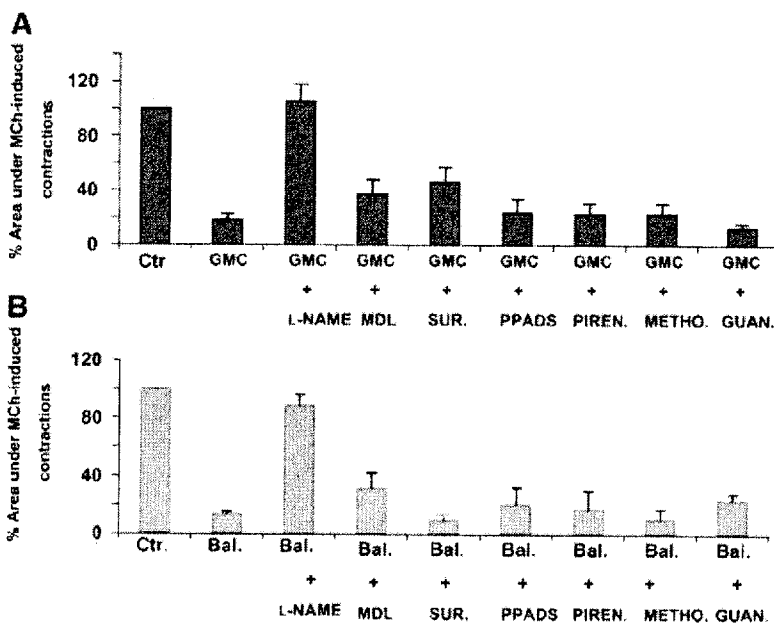Fig. 6. Bar graph showing the effects of L-NAME, MDL-12330 A (MDL), suramine, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic (PPADS), pirenzepine (Piren.), methoctramine (Metho.), and guanethidine (Guan.) on descending inhibition ahead of a distally propagating GMC (*A*) and that produced by balloon (Bal.) distension at a proximal site (*B*). Only the inhibition of NOS blocked the descending inhibition in both cases. *n* = 5 or 6.
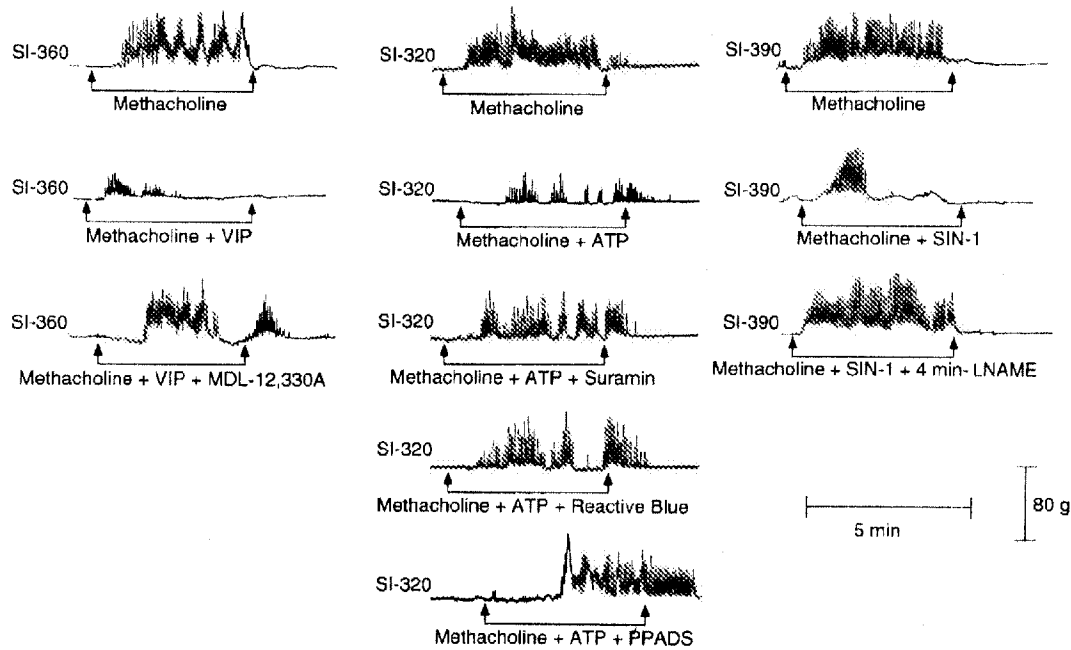
Fig. 7. Representative tracings that show the method to determine the effective doses of various antagonists. First, MCh was infused close intra-arterially as control to stimulate a series of RPCs. Then the inhibitory neurotransmitter was infused concurrently to show that it blocks the MCh-induced contractions. Finally, different concentrations of the receptor antagonists were infused at the same site. An effective concentration was that which reversed the inhibitory effect of the neurotransmitter. SIN-1,3-morpholinosydnoeimine.

decreased or 2) when they are accompanied with impaired descending inhibition so that the afferent signaling stimulated by distension of the distal segment produced by mass movement and that by compression of the proximal segment by the

GMC add up to a level above the nociceptive threshold (Fig. 9). Our findings show that when the contractility of the segment distended by a balloon is inhibited by atropine or verapamil, it does not stimulate afferent signaling.
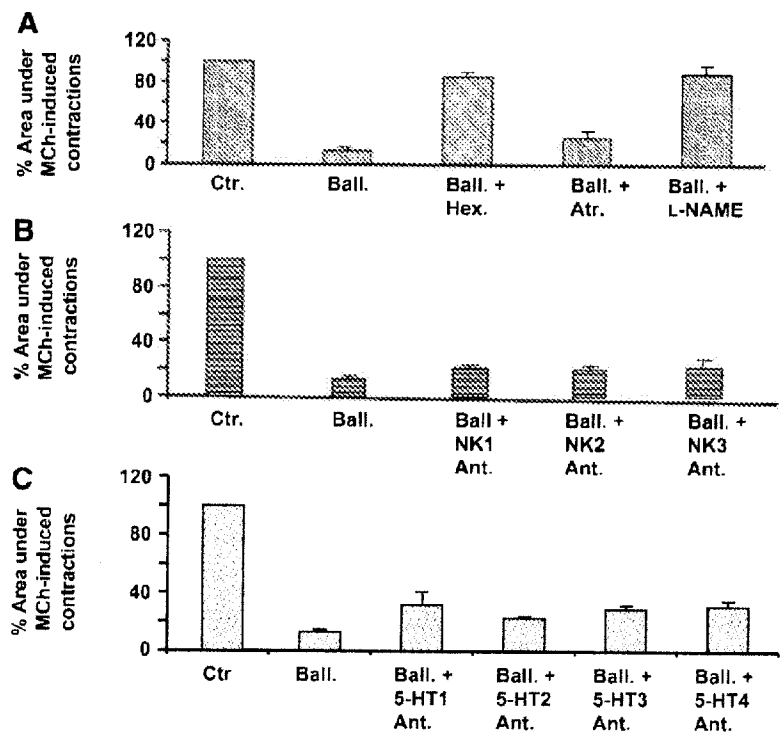
Fig. 8. Antagonists (Ant.) of muscarinic receptors, nicotinic receptors, NOS (A), NK_1, NK_2, NK_3 (B), 5-HT_{1A}, $5\text{-HT}_2/5\text{-}HT_{1C}$, and $5\text{-HT}_3/5\text{-HT}_4$ (C) were infused at the site of the middle catheter to determine whether they block descending inhibition produced by balloon distension at the proximal site. MCh was infused at the site of the distal catheter as control response to evaluate distending inhibition. Only the inhibition of nicotinic receptors and NOS blocked the descending inhibition (n = 5 or 6). Hex., hexamethonium.
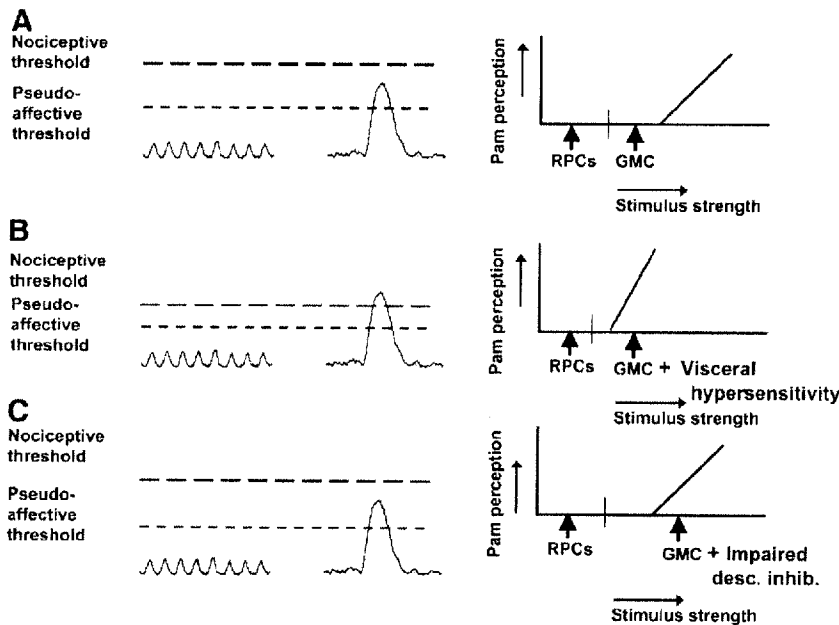
Fig. 9. Schematic illustration of the contributions of GMCs, visceral hypersensitivity, and impaired descending inhibition (desc. inhib.) to intermittent abdominal pain of gut origin. *A*: in normal health, the afferent signal strength stimulated by GMC exceeds the pseudo-affective threshold. The afferent signaling by RPCs is below this threshold. *B*: during visceral hypersensitivity, the nociceptive threshold is decreased, and therefore the afferent signaling by GMCs is perceived to be painful. *C*: during the impairment of descending inhibition, the afferent signaling due to strong compression of a proximal segment by a GMC and distension of the distal segment by large bolus add up to exceed the nociceptive threshold. Note that in some patients the amplitudes of GMCs have also been reported to increase. In these patients the afferent signaling due to these GMCs alone may exceed the nociceptive threshold. The vertical line between the RPCs and GMC indicates pseudoaffective threshold.

Some IBS patients do not demonstrate visceral hypersensitivity and yet they have the symptom of intermittent abdominal cramping (1). This can happen particularly if the internal anal sphincter or the ileocecal sphincter fails to relax ahead of a GMC and, therefore, not let the luminal contents pass through. Consequently, the segments proximal to them distend to add to the afferent signaling stimulated by the GMC itself. Therefore, a unifying hypothesis to explain the etiology of intermittent abdominal cramping may be that the occurrence of a GMC is the initiating event for nociceptive signaling, but it needs to be accompanied by visceral hypersensitivity and/or impaired descending inhibition for the signals to be perceived as painful.

The above unifying hypothesis is supported by several studies that show a correlation between the occurrences of GMCs in the small intestine or the colon and the sensation of intermittent pain in patients with IBS, IBD, or idiopathic constipation (2, 5, 13, 14, 44). On the other hand, the occurrence of GMCs in normal healthy subjects, who do not have visceral hypersensitivity or impaired descending inhibition, is not associated with pain. In normal healthy subjects, the occurrence and propagation of GMCs in the distal colon produces descending inhibition of the anal sphincter (37). It is also a common experience that abdominal pain occurs when defecation is withheld in the face of strong urge. A rapidly propagating GMC in the distal colon causes the urge to defecate because it pushes the fecal contents in the distal colon against closed anal sphincters and, therefore, it distends the anal canal (3, 24, 37). This pain is relieved upon defecation because the expulsion of feces eliminates the stimulus that initiates the GMCs. It is well established that the frequency of GMCs is increased in patients with diarrhea-predominant IBS and IBD (4, 13, 15, 41, 62), which is associated with an increase in the frequency of abdominal cramping.

Electrophysiological recordings from afferent neurons in anesthetized animals show that each contraction or distension in the gut stimulates afferent signaling whose intensity is proportional to the stimulus amplitude (8, 20, 61). It is likely that, because of their large amplitude and long duration, the GMCs trigger high-threshold sensory fibers, whereas the RPCs trigger low-threshold fibers (11). The central destinations of high- and low-threshold fibers may be different so that the activation of high-threshold fibers is perceived to be painful, whereas that of the low threshold fibers is not. The silent fibers that are activated in response to peripheral injury may also be triggered by large-amplitude GMCs to enhance afferent signaling in disease states (27).

The descending inhibition, a part of the peristaltic reflex, has been studied extensively in in vitro studies using flat sheet preparations and ex vivo segments (7, 34, 64). The relaxation of tone in a prestretched flat sheet or electrical recordings of inhibitory junction potentials were used as end points in response to muscle stretch, mucosal stroking, or balloon-induced distortion of flat sheets of gut segments in these studies. In our study, we used GMC, which occurs spontaneously but infrequently in the intact conscious state, as the stimulus. The descending inhibition in in vitro experiments is measured over 1- or 2-cm length. It is not known whether this inhibition extends over a sizable length of the gut, such as ~30 cm observed in the intact conscious state in our experiments.

The descending inhibition due to balloon distention was immediate, but that due to stimulation of a GMC was delayed until the GMC had spread over a sizable length of the intestine. The delay may be due to two reasons. First, that the GMC may take some time to spread over a sizeable length of intestine, which may be required to generate enough stimulus strength to induce descending inhibition. Our data with heart rate also showed that the GMC had to spread over a sizeable length of the intestinal segment before it could increase the heart rate. The second reason is that the inhibition of contractions in the segment ahead of a GMC may be to a limited length (30 cm in our study). Consequently, the GMC had to propagate within this distance of the distal segment to produce the inhibition of contractions in it.

Several neurotransmitters of the inhibitory motor neurons (e.g., NO, VIP and ATP) and of interneurons (tachykinins, serotonin, opioids, and other neuropeptides) and their receptors have been identified in the gut wall (26). A potential role of several of these mediators in descending inhibition has also been reported in in vitro preparations (9, 31, 33, 35, 64). However, our findings in the intact conscious state show that the synaptic transmission for descending inhibition is mediated primarily by nicotinic receptors and generation of NO in the interneurons, whereas the descending inhibition of smooth muscle contractions is mediated primarily by NO release from the inhibitory motor neurons. NO synthase has been identified in the interneurons (26); however, the mechanism by which NO regulates synaptic transmission for descending inhibition remains to be identified.

VIP inhibits smooth muscle contractions by the activation of adenylyl cyclase followed by generation of cAMP. We found that the inhibition of adenylate cyclase by MDL-12330A effectively blocked the inhibition of MCh-induced RPCs by exogenous VIP. However, this antagonist had only minor effect on balloon- or GMC-induced descending inhibition, whereas the inhibition of NOS with L-NAME blocked it almost completely. It is likely that the minor effect of VIP may be through the generation on NO as has been reported previously (32). Other investigators also failed to find a prominent role of endogenous VIP in inhibition of smooth muscle contractions (69) and concluded that its inhibitory effect depends on the experimental method used (23). By contrast, endogenous NO inhibits smooth muscle contractions in almost all experimental preparations.

In vitro studies show that the descending inhibition by muscle stretch is mediated by extrinsic neurons involving the prevertebral ganglia, whereas that induced by mucosal stroking is mediated by the descending interneurons (34). Our findings show that in the intact conscious state the descending inhibition by balloon distension that is equivalent to muscle stretch in vitro is mediated entirely by descending intrinsic neurons: the blockade of enteric nicotinic receptors by hexamethonium totally blocked this inhibition. Surgical myotomy also blocks descending inhibition (53).

In conclusion, GMCs, but not RPCs, stimulate afferent and descending neural signaling that produces pseudo-affective responses and descending inhibition, respectively. Numerous studies have reported the sensation of abdominal cramping with the occurrence of GMCs in IBS and IBD patients. The afferent signaling stimulated by GMCs in normal health is above pseudo-affective threshold, but it is subthreshold for nociception. However, if the sensory neurons are sensitized, as in IBS and IBD patients, to lower the nociceptive threshold, the same afferent signaling stimulated by GMCs may be perceived as painful. Alternately, if descending inhibition is impaired in a motility disorder, the afferent signaling due to strong compression of the intestine and distention of the distal receiving segment in the absence of relaxation of its tone and inhibition of spontaneous contractions add up to exceed the nociceptive threshold. The occurrence of GMCs, therefore, may be the central event to precipitate the sensation of intermittent abdominal cramping. The synaptic transmission to produce descending inhibition is mediated primarily by nicotinic receptors and release of NO, and inhibition of smooth muscle tone and spontaneous contractions is mediated by release of NO at the neuromuscular junction.

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Exhibit 4: Delafoy *et al.*, "Interactive Involvement of Brain Derived Neurotrophic Factor, Nerve Growth Factor, and Calcitonin Gene Related Peptide in Colonic Hypersensitivity in the Rat," *Gut* 55:940-945 (2006)

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NEUROGASTROENTEROLOGY

Interactive involvement of brain derived neurotrophic factor, nerve growth factor, and calcitonin gene related peptide in colonic hypersensitivity in the rat

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Background and aims: Neutrophins are involved in somatic and visceral hypersensitivity. The action of nerve growth factor (NGF) on sensory neurones contributes to the development of referred colonic hypersensitivity induced by trinitrobenzene sulfonic acid (TNBS). Based on data on brain derived neurotrophic factor (BDNF) and calcitonin gene related peptide (CGRP) in pain, the aims of the present study were: (1) to investigate the involvement of BDNF and CGRP in this model of referred colonic hypersensitivity, (2) to test the effect of exogenous BDNF and CGRP on the colonic pain threshold, and (3) to investigate the relationship between BDNF, NGF, and CGRP by testing antineurotrophin antibodies or h-CGRP 8–37 (a CGRP antagonist) on bowel hypersensitivity induced by these peptides.
Methods: Colonic sensitivity was assessed using a colonic distension procedure.
Results: Anti-BDNF antibody and h-CGRP 8–37 reversed the induced decrease in colonic threshold (33.4 (2.1) and 40.3 (4.1) mm Hg, respectively, compared with a vehicle score of approximately 18 mm Hg; p<0.001). BDNF (1–100 ng/rat intraperitoneally) induced a significant dose dependent decrease in colonic reaction threshold in healthy rats. This effect was reversed by an anti-BDNF antibody and an anti-NGF antibody (33.4 (0.6) v 18.7 (0.7) mm Hg (p<0.001), anti-NGF v vehicle). NGF induced colonic hypersensitivity was reversed by h-CGRP 8–37 but not by the anti-BDNF antibody. Finally, antineurotrophin antibody could not reverse CGRP induced colonic hypersensitivity (at a dose of 1 μg/kg intraperitoneally).
Conclusion: Systemic BDNF, NGF, and CGRP can induce visceral hypersensitivity alone and interactively. This cascade might be involved in TNBS induced referred colonic hypersensitivity in which each of these peptides is involved.

Functional digestive disorders are often associated with a decrease in visceral pain threshold indicating visceral hypersensitivity.[1][2] Indeed, patients suffering from irritable bowel syndrome (IBS) demonstrate a lower visceral sensory threshold to colorectal balloon distension.[3] It has been suggested in IBS that there is heightened pain sensitivity of the brain-gut axis, with a normal pattern of activation.[2] We have previously shown that injection of trinitrobenzene sulfonic acid (TNBS) into the proximal colon provoked chronic colonic hypersensitivity, measured in conscious rats by a decreased pain threshold in response to colonic distension. This chronic hypersensitivity was found in the distal non-inflamed colon and persisted for 21 days.[4] It mimicked certain characteristics of IBS and so it can be used as a model to experimentally explore the pathophysiological aspects of this disorder. We have previously shown that the action of nerve growth factor (NGF) on sensory neurones contributes to the development of visceral hypersensitivity in this model.[5] NGF induced colonic hypersensitivity, which was prevented by an anti-NGF antibody, was also able to prevent TNBS induced referred non-inflammatory colonic hypersensitivity.

Brain derived neurotrophic factor (BDNF) is a type of neurotrophin which has been studied for its role in neuronal survival and development. Recently, much attention has focused on the role of BDNF as a neuromodulator, especially in inflammatory pain states. In humans, it is now known that BDNF is upregulated and associated with pain in chronic

pancreatitis.[6][7] In animals, levels of BDNF or TrkB receptor were increased in models of bladder inflammation[7] and nerve injury.[8][9] These increases were associated with pain behaviour.[10] Data on the effects of exogenous BDNF on pain remain inconclusive but several studies have shown that BDNF has nociceptive effects.[11][12] Finally, BDNF has been shown to be involved in the pathophysiology of pain via sequestration. Anti-BDNF antibody relieved mechanical or thermal hyperalgesia in rat models of nerve ligation.[4][13] The sequestering antibody trkB-IgG significantly reduced behavioural nociceptive responses evoked after subcutaneous injection of dilute formalin or carrageenan into one hind paw.[14–16] Antibodies against different neurotrophins (NGF, BDNF, and neurotrophin 3), delivered directly to the injured dorsal root ganglia, significantly reduced (with different time sequences) the percentage of paw withdrawal responses evoked by von Frey hairs after spinal nerve injury.[12] Taken together, these data suggest that BDNF seems to be involved in the mechanism of hyperalgesia.

It is now well established that calcitonin gene related peptide (CGRP) is implicated in several models of visceral pain.[17–26] CGRP is by far the most abundant peptide of

capsaicin sensitive afferent fibres of gastrointestinal origin, accounting for up to 80% of overall peptide immunoreactivity.[21 22] Recently, morphological colonic distribution of CGRP and substance P (SP) immunoreactive nerves was investigated in our model of TNBS induced referred non-inflammatory colonic hypersensitivity.[23] This study showed that the inflammatory process in the proximal colon induced, at a distance (that is, in the distal colon), a highly significant increase in SP and CGRP innervation of the myenteric plexus.[23]

Based on data on BDNF, CGRP, and NGF on pain and, for NGF, on the model of TNBS induced referred non-inflammatory colonic hypersensitivity, the aims of the present study were to determine the involvement of BDNF and CGRP in the same model, and to test the link between NGF, BDNF, and CGRP to determine their respective involvement in the peripheral pathophysiology of non-inflammatory colonic hypersensitivity.

METHODS
Animals
This study was carried out on adult male Wistar rats (Janvier, Le Genest-St-Isle, France) weighing 320–350 g. Animals were housed three per cage under conditions of controlled temperature ($20 \pm 1°C$), hygrometry ($50 \pm 5\%$), and lighting (lights on from 7am to 7pm) for at least one week prior to the experiments. They were deprived of food for 18 hours but allowed access to water ad libitum up to the start of the experiments. All studies were performed in accordance with the ethical guidelines of the International Association for the Study of Pain.[24] Great care was taken, particularly with regard to housing conditions, to avoid or minimise discomfort to the animals.

Assessment of colonic sensitivity
A latex balloon (5 cm length) was inserted through the anus and placed in the distal colon, 5 cm from the anus. To maintain this position, the catheter was taped onto the base of the tail. Animals were individually placed without restraint in polypropylene cages for the distension session. After a 30 minute acclimatisation period, the balloon was progressively inflated from 0 to 75 mm Hg, in 5 mm Hg increments, every 30 seconds. Each cycle of colonic distension was controlled by an electronic barostat (ABS, St-Dié, France). The colonic reaction threshold was determined as the pressure inducing the first abdominal contraction and consequently interruption of the cycle. Abdominal contraction corresponds to waves of contraction of oblique musculature with inward turning of the hind limb, to the hump backed position, or to squashing of the lower abdomen against the floor.[25] Similar behaviours have previously been used as colonic reaction thresholds to noxious stimuli.[26-28] To average the colonic reaction threshold, each rat was subjected to four distension cycles (D1–D4) with a 10 minute interval between each cycle. At the end of the four cycles, animals were sacrificed by cervical dislocation.

Induction of colonic hypersensitivity
Under anaesthesia (acepromazine 12 mg/kg intraperitoneally and ketamine 80 mg/kg intraperitoneally), injection of TNBS, dissolved in 30% ethanol, was given at 50 mg/kg (1.5 ml/kg) into the proximal colon, 1 cm from the caecum. Following administration, rats were individually housed in polypropylene cages and maintained for seven days under the controlled conditions described above. Corresponding controls were healthy animals housed under the same conditions. It has previously been shown that sham operated animals had no pathophysiological signs of visceral inflam-

mation in both proximal- and distal colon-like saline or EtOH 30% injected animals.[4] Mean weight of animals before injury was of 361 (4) g; three days after injury mean weight was 348 (5) g and seven days after injury mean weight was 349 (7) g.

Experimental protocol
Three series of experiments were conducted. For each treated group, n = 7 or 8 animals were used.

To determine the involvement of BDNF and CGRP in the TNBS model of referred non-inflammatory colonic hypersensitivity, the first series of experiments were conducted on seven groups of TNBS treated rats (50 mg/kg into the proximal colon), seven days before distension, and in one group of healthy rats without any intracolonic administration. Three groups of TNBS treated rats received anti-BDNF antibody (36 μg/kg) or its vehicle (bovine serum albumin (BSA) 0.1%), or a control isotype antibody (40 μg/kg) intraperitoneally, 30 minutes before distension. Four other groups of TNBS treated rats received h-CGRP 8–37, a CGRP receptor antagonist, at a dose of 75, 150, or 225 μg/kg, or its vehicle (BSA 0.1%) intravenously, 30 minutes before distension.

In the second series of experiments, the effect of exogenous BDNF was tested to confirm its ability to induce colonic hypersensitivity. Four groups of rats received BDNF in 0.1% BSA at doses of 1, 10, or 100 ng /rat, or its vehicle (0.1% BSA), intraperitoneally (0.5 ml/rat), 30 minutes before distension.

The third series of experiments was performed to test the reciprocal influence of an anti-BDNF or anti-NGF antibody, or of h-CGRP 8–37, on hypersensitivity induced by BDNF, NGF, or CGRP. In the initial experiment, the effect of an anti-BDNF and anti-NGF antibody on BDNF induced hypersensitivity was studied. Administration of the antibody and peptide was quasi-simultaneous, both intraperitoneally, 30 minutes before distension. The control group received 0.1% BSA or anti-BNDF antibody (36 μg/kg) while the other groups received 100 ng BDNF/rat with either the anti-BDNF (18 or 36 μg/kg or control isotype antibody 40 μg/kg) or anti-NGF antibody (1/2000, 2 ml/kg, a dose which blocks the hyperalgesic effect of NGF[5]), or vehicle.

The aim of the second experiment was to test the effect of anti-BDNF antibody and h-CGRP 8–37 on NGF induced hypersensitivity. The control group received NGF vehicle (BSA 0.1%) whereas the other three groups were treated with NGF (10 ng/rat intraperitoneally) and received the anti-BDNF antibody (36 μg/kg intraperitoneally), h-CGRP 8–37 (300 μg/kg intravenously, a dose which blocks the hyperalgesic effect of CGRP[23]), or vehicle, 30 minutes before distension.

Finally, in a third experiment, we studied the effect of anti-NGF and anti-BDNF antibodies on CGRP induced hypersensitivity. The control group received CGRP vehicle (BSA 0.1%) whereas the other three groups were treated with CGRP (1 μg/kg intraperitoneally) and received the anti-NGF (1/2000, 2 ml/kg intraperitoneally) or anti-BDNF antibody (36 μg/kg intraperitoneally), or vehicle (BSA 0.1%), 30 minutes before distension.

All the experiments were performed blind by the same investigators. Treatments were administered randomly. Different animals were used for each experimental study.

Compounds
BDNF was a human recombinant form (expressed in Escherichia coli) and NGF-2.5S was obtained from mouse submaxillary gland. Monoclonal antihuman BDNF (IgG1 isotype) was purified from a mouse hybridoma. The corresponding control isotype antibody was a purified mouse
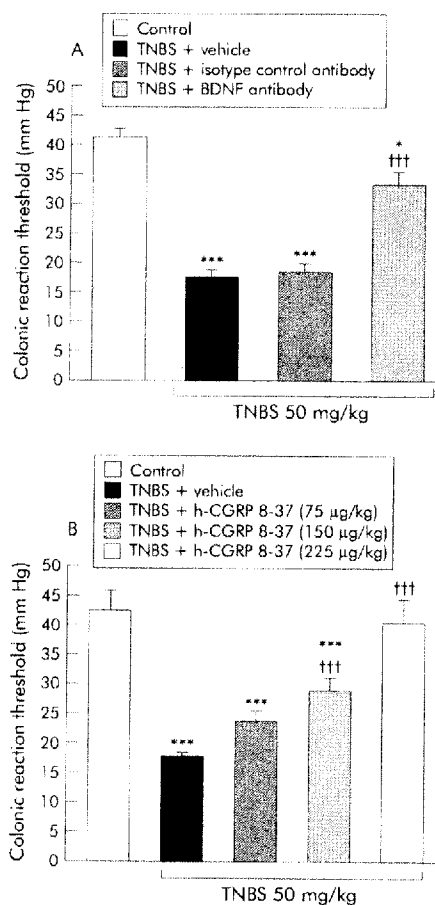
Figure 1 Effect of an anti-brain derived neurotrophic factor (BDNF) antibody (36 µg/kg intraperitoneally 30 minutes before distension), its vehicle (bovine serum albumin (BSA) 0.1%), a control isotype antibody (40 µg/kg) (A), or human calcitonin gene related and peptide fragment 8–37 (h-CGRP 8–37) antagonist (75, 150, and 225 µg/kg, intravenously, 30 minutes before distension) (B) on colonic reaction threshold of trinitrobenzene sulfonic acid (TNBS) treated rats (50 mg/kg, 30% ethanol, into the proximal colon, seven days before distension), in response to colonic distension in the distal colon. Results are expressed as mean (SEM) (n = 7–8 per group). *p<0.05, ***p<0.001 versus control group; †††p<0.001 versus colonic reaction threshold of TNBS treated rats + vehicle.
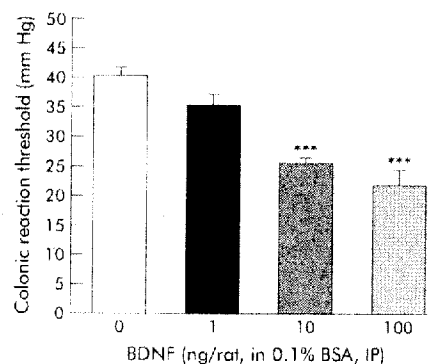

Figure 2 Effect of exogenous brain derived neurotrophic factor (BDNF) (0–100 ng/rat in 0.1% bovine serum albumin (BSA), intraperitoneally (IP)) on colonic reaction threshold of rats in response to colonic distension. Results are expressed as mean (SEM) (mm Hg) (n = 7–8 per group). ***p<0.001 versus vehicle treated control group.

IgG1. Polyclonal neutralising NGF antibody was developed in rabbit against NGF-2.5S from mice. The corresponding control antibody was a rabbit IgG. Calcitonin gene related peptide (α-CGRP, human) was of synthetic origin, as was the human calcitonin gene related and peptide fragment 8–37 (h-CGRP 8–37), a selective peptidic CGRP1 receptor antagonist.[29-31] TNBS was purchased from Fluka (Buchs, Switzerland) and was dissolved in 30% EtOH. Other compounds were supplied by Sigma-Aldrich Chemical Co (St Louis, Missouri, USA) and were dissolved in BSA 0.1%.

Expression of results and statistical analysis

Results are expressed as mean (SEM) of balloon pressure in mm Hg that induce the first abdominal contraction. Statistical comparisons between the different groups were made using one way ANOVA followed by a Bonferroni post hoc test to compare several treatments. Differences were considered statistically significant at p<0.05.

RESULTS
Evaluation of the involvement of BDNF and CGRP in TNBS induced referred colonic hypersensitivity

Rats treated with TNBS (50 mg/kg into the proximal colon) seven days before distension had a significant decrease in colonic reaction threshold in response to distal colonic distension (18.6 (1.3) v 41.3 (1.8) mm Hg for controls; p<0.001) (fig 1A). Administration of anti-BDNF antibody (36 µg/kg intraperitoneally), which has no effect in healthy rats, produced a colonic reaction threshold of 41.4 (1.8) mm Hg, which was not significantly different from control colonic reaction thresholds of healthy rats (41.2 (1.35) mm Hg). The same dose of anti-BDNF antibody significantly reduced (but not totally reversed) TNBS induced referred non-inflammatory colonic hypersensitivity (33.4 (2.1) v 18.6 (1.3) mm Hg (p<0.001), anti-BDNF v vehicle). The isotype control antibody had no effect on this decrease. h-CGRP 8–37 inhibited the decrease in reaction threshold in a dose dependent manner: at a dose of 225 µg/kg, inhibition was maximal with a colonic reaction threshold of 40.3 (4.1) mm Hg compared with that of TNBS treated rats injected with vehicle (17.5 (0.7) mm Hg; p<0.001) or healthy control rats (42.4 (3.2) mm Hg) (fig 1B).

Effect of BDNF on pain threshold in response to colonic distension

The colonic pain threshold of control rats, treated with vehicle only (0.1% BSA), was 40.4 (1.2) mm Hg. Intraperitoneal injection of BDNF in 0.1% BSA (1–100 ng/rat), 30 minutes before distension, induced a significant dose dependent decrease in colonic pain threshold (fig 2). With a dose of 1 ng/rat, the decrease was not statistically significant compared with control rats (35.4 (1.8) v 40.4 (1.2) mm Hg, respectively). For BDNF at 10 and 100 ng/rat, the colonic pain threshold was 21.5 (2.7) and 25.5 (2.1) mm Hg (p<0.001 v control threshold). The dose of 100 ng BDNF/rat was chosen for the following experiments.

Reciprocal influence of neurotrophin antibodies or CGRP antagonist on hypersensitivity induced by various peptides
Effect of anti-BDNF and anti-NGF antibody on pain threshold in response to colonic distension of BDNF treated rats

Anti-BDNF antibody (36 µg/kg intraperitoneally) reversed the decrease in colonic pain threshold induced by simultaneous intraperitoneal injection of BDNF (100 ng/rat)
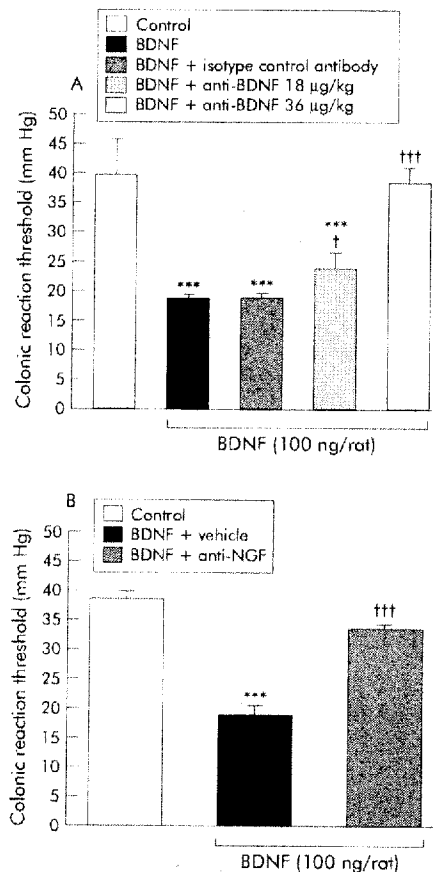
Figure 3 Effect of an anti-brain derived neurotrophic factor (BDNF) antibody (0, 18, or 36 μg/kg in bovine serum albumin (BSA) 0.1% intraperitoneally) (A) and an anti-nerve growth factor (NGF) antibody (1/2000, 2 ml/kg intraperitoneally) (B) on colonic distension threshold of BDNF treated rats (100 ng/rat in 0.1% BSA intraperitoneally). Results are expressed as mean (SEM) (mm Hg) (n = 7–8 per group). ***p<0.001 versus control group; † p<0.05; †††p<0.001 versus colonic reaction threshold of BDNF treated rats + vehicle.

Figure 4 Effect of an anti-brain derived neurotrophic factor (BDNF) antibody (36 μg/kg intraperitoneally in bovine serum albumin (BSA) 0.1%) and human calcitonin gene related and peptide fragment 8–37 (h-CGRP8-37) antagonist (300 μg/kg intravenously) on colonic reaction threshold of nerve growth factor (NGF) treated rats (10 ng/rat, in 0.1% BSA intraperitoneally). Results are expressed as mean (SEM) (mm Hg) (n = 7–8 per group). ***p<0.001 versus control group; †††p<0.001 versus colonic reaction threshold of NGF treated rats + vehicle.

p<0.01). Colonic reaction threshold of the h-CGRP8–37 treated group was not statistically different from the control threshold (without NGF).

Effect of anti-NGF and anti-BDNF on pain threshold in response to colonic distension of CGRP treated rats

Rats treated with CGRP (1 μg/kg intraperitoneally) had a significant decrease in pain threshold in response to colonic distension (21.0 (1.5) v 39.7 (2.1) mm Hg for the control group; p<0.001) (fig 5). Neither anti-NGF (1/2000, 2 ml/kg intraperitoneally) nor anti-BDNF antibody (36 μg/kg intraperitoneally) was able to reverse this CGRP induced decrease.

DISCUSSION

We have previously shown that NGF contributes, via capsaicin sensitive fibres, to the development of TNBS induced referred colonic hypersensitivity in response to distal

(38.1 (2.6) v 18.6 (0.8) mm Hg; p<0.001) for BDNF treated rats receiving vehicle (fig 3A). The threshold obtained for the anti-BDNF treated group was not significantly different from the control threshold. Half the dose of anti-BDNF antibody had a small effect on this decrease. Colonic pain threshold was similar in isotype control antibody treated rats and vehicle treated rats. Similarly, anti-NGF antibody (1/2000, 2 ml/kg intraperitoneally) inhibited the BDNF induced decrease in colonic pain threshold (33.4 (0.6) v 18.7 (0.7) mm Hg; p<0.001) for BDNF treated rats receiving vehicle (fig 3B). Again, the threshold obtained for the anti-NGF treated group was not significantly different from the control threshold (without BDNF).

Effect of anti-BDNF and h-CGRP8–37 on pain threshold in response to colonic distension of NGF treated rats

Rats treated with NGF (10 ng/rat intraperitoneally) had a significant decrease in pain threshold in response to colonic distension (18.2 (1.1) mm Hg v 40.3 (0.9) mm Hg for the control group; p<0.001) (fig 4). The anti-BDNF antibody (36 μg/kg intraperitoneally) was unable to reverse this decrease. On the other hand, h-CGRP8–37 (300 μg/kg intravenously) reversed NGF induced colonic hypersensitivity (32.1 (3.6) mm Hg v vehicle treated rats 18.2 (1.1) mm Hg;
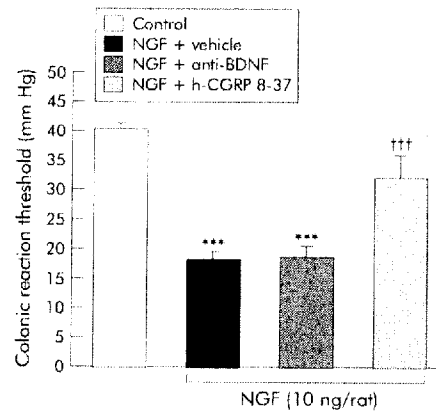

Figure 5 Effect of an anti-brain derived neurotrophic factor (BDNF) antibody (36 μg/kg intraperitoneally) and an anti-nerve growth factor (NGF) antibody (1/2000, 2 ml/kg intraperitoneally) on colonic reaction threshold of calcitonin gene related peptide (CGRP) treated rats (1 μg/kg in 0.1% bovine serum albumin intraperitoneally). Results are expressed as mean (SEM) (mm Hg) (n = 7–8 per group). *p<0.05; ***p<0.001 versus control group; NS, non-significant difference from colonic reaction threshold of CGRP treated rats + vehicle.

colonic distension, a model that can mimic IBS in rats.[4 5] In our study, this model was used to test the same hypothesis with other neurotrophins, BDNF and CGRP, and the relationship between BDNF, NGF, and CGRP in colonic hypersensitivity was also investigated.

Our main findings were that: (1) anti-BDNF antibody and CGRP antagonist alleviated TNBS induced referred colonic hypersensitivity, which demonstrates (in common with NGF) involvement of this neurotrophin and this neuropeptide in the pathophysiology of a model which mimics certain aspects of IBS; (2) confirmation that BDNF and CGRP induce colonic hypersensitivity; and (3) BDNF, NGF, and CGRP can induce colonic hypersensitivity in an interactive manner.

Take together, the ability of anti-BDNF antibody to markedly reduce the TNBS induced decrease in distal colonic distension thresholds and its inability to modify this parameter in healthy rats demonstrates that the neurotrophin is involved in colonic hypersensitivity. This conclusion is in agreement with the demonstrated hyperalgesic effect of low doses of exogenous BDNF which, in terms of magnitude and onset, are similar to that induced by exogenous NGF in the colorectal distension test.[5] It is also in accordance with previous studies demonstrating a nociceptive effect of exogenous BDNF in various models. Exogenous BDNF, like exogenous NGF, triggered a persistent mechanical allodynia when delivered directly to the intact dorsal root ganglia.[12] Injection of BDNF into the rat hind paw has been shown to induce thermal hyperalgesia.[11] These data are also in accordance with other studies where BDNF mRNA and protein expression were upregulated in some models of inflammatory and neuropathic pain.[8 9 32-34]

BDNF and NGF are thought to be involved in long term plasticity.[35 36] However, in our studies (the present work and Delafoy and colleagues[5]), seven days after injury (TNBS in the proximal colon), a single injection of antibodies was effective at the same dose as that used to reverse the acute hyperalgesic effect of their respective neurotrophins, suggesting ongoing release of the two neurotrophins in non-inflammatory colonic hypersensitivity. Moreover, it is important to note that anti-BDNF and anti-NGF[5] antibodies did not completely reverse this hypersensitivity. Accordingly, we hypothesise that two mechanisms may be involved in neurotrophin mediated non-inflammatory colonic hypersensitivity. The first mechanism, involving early released neurotrophins inducing long term plastic changes insensitive to acutely administered antibodies injected several days after injury, and a second one (predominant) involving ongoing release of neurotrophins inducing hyperalgesic effects which can be reversed by antibodies. The suspected ongoing release of neurotrophin is in agreement with some of the data in the literature. In a model of visceral hypersensitivity induced by neonatal maternal deprivation, adult deprived rats treated with anti-NGF antibodies exhibited normal gut permeability and visceral sensitivity to rectal distension.[37] Anti-BDNF antibody, injected three days after injury, has been shown to relieve mechanical or thermal hyperalgesia in rat models of nerve ligation.[8 13] Antibodies to different neurotrophins (NGF, BDNF, neurotrophin 3) significantly reduced the percentage of foot withdrawal responses evoked by von Frey hairs when injected up to 14 days after spinal nerve injury.[12] Moreover, findings of an increase in expression of neurotrophins, in visceral pain models or in patients, indirectly suggest ongoing involvement of neurotrophins. In a model of colonic hypersensitivity induced by neonatal stress in rats, NGF expression (protein and mRNA) increased for up to 12 weeks of life.[37] In *Trichinella spiralis* infected rats, which provide a model of small intestine hypersensitivity, NGF protein and mRNA levels were significantly increased in intestine tissue three days post infection.[38] In inflamed gut of

patients with colitis, tissues are rich in mast cells and high levels of NGF were observed.[39] BDNF is also upregulated and associated with pain in patients with chronic pancreatitis.[6]

Moreover, the data obtained in this study provide further insight into the "hypersensitising" action of BDNF. The efficacy of low systemic doses of BDNF, its short delay of action in inducing a decrease in distension thresholds, and its inability to cross the blood-brain barrier[40] suggest a peripheral action of this neurotrophin. However, release of endogenous BDNF and the mechanisms by which it occurs, in our model of colonic hypersensitivity, remains to be determined.

The pronociceptive role of CGRP has been well established.[17-20 41] Accordingly, in our study, as with BDNF, we have shown the ability of CGRP to induce colonic hypersensitivity and its involvement in TNBS induced referred colonic hypersensitivity. This latter result is also consistent with the previously described peripheral involvement of CGRP in the mechanism of neurogenic inflammation,[35 42] which could be the case here.

Another major finding of this study was that involvement of BNDF, NGF, and CGRP in induction of non-inflammatory colonic hypersensitivity is linked. One hypothesis would be an interactive involvement according to an ordered cascade. Indeed, in our study, the anti-BDNF antibody only reversed BDNF induced hypersensitivity and was inactive on both NGF and CGRP induced hypersensitivity. Secondly, BDNF induced hypersensitivity was reversed by the anti-NGF antibody. Thirdly, NGF induced hypersensitivity was alleviated only by h-CGRP 8–37. Finally, none of the anti-neurotrophin antibodies tested was able to reduce CGRP induced hypersensitivity. Taken together, these results suggest that the cascade is as follows: first BDNF, which needs the involvement of NGF, which in turn needs CGRP to induce colonic hypersensitivity. This cascade is supported by the fact that anti-BDNF, anti-NGF, and CGRP antagonist reversed TNBS induced referred colonic hypersensitivity by 65%, 86%, and 93%, respectively. We may hypothesise that TNBS induces release of neurotrophins from peritoneal mast cells, for example,[39 41] in first BDNF and then NGF, which in turn triggers release of CGRP from capsaicin sensitive primary sensory afferents. This is in agreement with: (i) our previous demonstration that neonatal treatment with capsaicin reduces NGF and TNBS induced hypersensitivity, suggesting that capsaicin sensitive primary afferents are required in the development of colonic hypersensitivity[5]; (ii) the fact that CGRP is present in these sensory afferent fibres[44-46] and that capsaicin, which induces depletion of CGRP,[47] also reduces, for example, peritoneal irritation induced visceral pain[17 19 20]; and (iii) the fact that NGF has been shown to be involved in CGRP secretion in sensitive neurones[28 48-51] and to regulate expression of neuropeptide genes in adult sensory neurones.[52] Recent work shows that NGF increases CGRP release due to TRPV1 agonists, such as anandamide.[53]

Accordingly, we know that in our model of colonic hypersensitivity there is a highly significant increase in SP and CGRP innervation of the myenteric plexus in the distal colon.[23] However, regarding partial inhibition of TNBS induced referred colonic hypersensitivity by neurotrophin antibodies, we may also speculate that other pathways, neurotrophin independent, could be involved in CGRP receptor involvement. Indeed, the existence of the suspected cascade needs to be confirmed (for example, study of the time course involvement of the various mediators) and the link between these three mediators may also involve another mechanism, such as a synergistic interaction.

In conclusion, our results highlight the importance of BDNF and CGRP, as previously shown for NGF, in

non-inflammatory colonic hypersensitivity and demonstrate their interactive involvement. Due to the similar characteristics of the animal model used with IBS, these findings can help in understanding the pathophysiology of IBS and may offer interesting pharmacological perspectives.

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Exhibit 5: Bourdu *et al.*, "Rectal Instillation of Butyrate Provide Novel Clinically Relevant Model of Noninflammatory Colonic Hypersensitivity," *Gastroenterology* 128:1996-2008 (2005)

Rectal Instillation of Butyrate Provides a Novel Clinically Relevant Model of Noninflammatory Colonic Hypersensitivity in Rats

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Background & Aims: The treatment of irritable bowel syndrome (IBS), characterized by abdominal pain and bloating, is empirical and often poorly efficient. Research lacks suitable models for studying the pathophysiologic mechanisms of the colonic hypersensitivity and new pharmacologic targets. The present study aimed to develop a novel model of colonic hypersensitivity possessing several of the characteristics encountered in patients with IBS. *Methods:* Rats received enemas of a butyrate solution (8–1000 mmol/L) twice daily for 3 days. A time course was determined for colonic hypersensitivity (colorectal distention test) and referred cutaneous lumbar hyperalgesia (von Frey hairs). Macroscopic and histologic analyses were performed on colonic mucosa. The efficacy of morphine, U50488H (a κ opioid agonist), and trimebutine on the 2 pain parameters was determined. Finally, the involvement of peptidergic C-fibers was evaluated using capsaicin-pretreated animals and treatments with calcitonin gene-related peptide (CGRP) and neurokinin 1 receptor antagonists. *Results:* Butyrate enemas induced a sustained, concentration-dependent colonic hypersensitivity and, to a lesser extent, a referred cutaneous mechanical hyperalgesia, particularly in female rats, but no macroscopic and histologic modifications of the colonic mucosa, as observed in patients with IBS. Both pain parameters were sensitive to morphine, U50488H, trimebutine, neonatal capsaicin treatment, and the CGRP receptor antagonist but not to the neurokinin 1 receptor antagonist. *Conclusions:* These results present our noninflammatory model of chronic colonic hypersensitivity as a useful novel tool for studying IBS. The CGRP receptor antagonist-induced reduction of colonic hypersensitivity suggests that CGRP receptors may provide a promising target for treatment of IBS.

I rritable bowel syndrome (IBS) is nowadays a health care burden, because it represents one of the most common disorders encountered in gastrointestinal prac-

tice and highly affects quality of life.[1] The major features for diagnosing IBS have been defined as (1) abdominal pain or discomfort, (2) altered bowel habits, and (3) the absence of any detectable structural or biochemical abnormality.[2] Chronic or recurrent abdominal pain represents a symptom common to all patients with IBS, and rectal or colonic hypersensitivity is frequently encountered in IBS.[1,3] The underlying causes of the pathophysiologic changes responsible for these changes in colonic sensitivity remain unclear. Animal models must therefore be developed to investigate the pathophysiologic mechanisms underlying the colonic hypersensitivity observed in IBS,[4] which could lead to more adapted treatments because current treatments have thus far proved unsatisfactory. Some models have been developed to obtain colonic hypersensitivity in adult rats after colonic irritation either induced by neonatal repeated colonic distention or inflammation[5] or following repeated neonatal stressful separation of rat pups from their mother.[6] Diop et al[7] developed a model in adult rats in which distant colonic hypersensitivity (distal part of the colon) was induced using an injection of trinitrobenzene sulfonic acid (TNBS) in the proximal part. Recently, we showed a visceral hypersensitivity in rats elicited by enemas with butyrate, a short-chain fatty acid produced by the colonic degradation of alimentary fibers.[8] This is in line with the observation that colonic butyrate levels were increased in patients with IBS.[9] These observations argue for the use of butyrate as an etiologic factor, in link with nutrition, to create a novel, particularly relevant

animal model of colonic hypersensitivity as observed in patients with IBS.

The aim of this study was first to characterize whether butyrate enemas could provoke a distention-induced colonic hypersensitivity in rats and/or an alteration of bowel habits and if these modifications were independent of any detectable abnormality of the mucosa as observed in patients with IBS.[10] Hence, macroscopic, microscopic, or biochemical modifications of the colon mucosa were evaluated in our experimental conditions.

After having determined the characteristics and clinical relevance of our model, it was important to test its pharmacologic sensitivity to both reference analgesic drugs such as opioids and compounds such as trimebutine maleate, which is commonly used in patients with IBS.

One factor that could explain the difficulty in proposing new treatments in patients with IBS is the poor understanding of the pathophysiologic mechanisms involved in the development of this pathology. Hence, using this model, we looked to research the pathophysiologic mechanisms that could be involved in noninflammatory colonic hypersensitivity. We chose to determine whether peptidergic C-fibers were involved because these fibers are known to be involved in several chronic pain contexts, including neuropathic[11,12] and inflammatory[13,14] pain, and also in bronchial hypersensitivity.[15] This involvement was assessed using neonatal capsaicin-treated animals; in fact, both peptidergic C-fibers, which synthesize and release substance P and calcitonin gene-related peptide (CGRP), and nonpeptidergic C-fibers, which do not express these peptides, are sensitive to capsaicin.[16] Thus, to show the involvement of the peptidergic C-fiber population, we studied the involvement of the major peptides peripherally released from these fibers, substance P, and CGRP. The role of substance P has been demonstrated in several inflammatory bowel models[17,18] or in acute visceral hyperalgesia.[19] Moreover, substance P receptors (neurokinin 1 [NK1] receptors) have been proposed as targets for IBS treatment.[20,21] We also chose to investigate the involvement of CGRP, which is colocalized with substance P in peptidergic C-fibers and has been poorly investigated in the context of colonic hypersensitivity.

Materials and Methods

Animals

Male and female Sprague–Dawley rats (Charles River, L'Arbresle, France) weighing 200–220 g were used in this study. Rats were maintained in laboratory conditions for 1 week before each experiment. The animals were housed 5 per cage with food and water available ad libitum. All studies were

performed in accordance with the proposal of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.[22] Great care was taken, particularly with regard to housing conditions, to avoid or minimize discomfort to the animals.

Induction of Colonic Hypersensitivity

Butyrate enemas. For each enema, a catheter (2-mm Fogarty catheter) was placed into the colon at 7 cm from the anus, and the animals received 1 mL of sodium butyrate solution at neutral pH (pH 6.9) twice daily for 3 days.

TNBS enema. A TNBS model was used as a positive control for inflammation. Colitis was induced under anesthesia (acepromazine 4 mg/kg intraperitoneally, ketamine 30 mg/kg intraperitoneally) in 24-hour fasted rats by a single intracolonic enema with 0.5 mL of 50 mg/kg TNBS dissolved in 30% ethanol solution. TNBS was introduced through a 7-cm-long Fogarty probe inserted into the descending colon.

Behavioral Testing

Evaluation of colonic sensitivity. Nociception in the animals was assessed by measuring the intracolonic pressure required to induce a behavioral response during colorectal distention (CRD) due to the inflation of a balloon introduced in the colon. This response was characterized by an elevation of the hind part of the animal body and a clearly visible abdominal contraction corresponding to the severe contractions described by Al Chaer et al[5] and used as a pain marker by Tarrerias et al.[8] This method was chosen to avoid the surgery needed to implant recording electrodes and wires in the abdominal muscles, which may cause additional sensitization and disrupt the evaluation of lumbar cutaneous referred hyperalgesia. Distention balloons were prepared using a 2-mm Fogarty catheter cut at 9 cm. A 2-cm flexible latex balloon was ligated to the tip of the catheter. Rats were anesthetized with volatile anesthesia (2% isoflurane), the balloon was inserted intrarectally in a minimally invasive manner to 7 cm from the anus, and the catheter was taped to the base of the tail. After 5 minutes, rats were placed in the middle of a 40 × 40-cm Plexiglas box and the catheter was connected to an electronic barostat apparatus (Synectics Visceral Stimulator; Medtronic, Boulogne-Billancourt, France). Increasing pressure was continuously applied until pain behavior was displayed or a cutoff pressure of 80 mm Hg was reached. The influence of volatile anesthesia on motor activity (Actimeter Apelex panlab 0602 and 0603; Apelex, Massy, France) was controlled to make sure that there was no residual effect on the motor reaction of animals to CRD. There was no difference in the spontaneous motor activity of nonanesthetized and anesthetized rats (453 ± 22.53 vs 406 ± 20.17 arbitrary units, respectively) between 5 and 10 minutes following isoflurane exposure.

To confirm the butyrate-induced colonic hypersensitivity, another pain evaluation method was used. Using the same protocol for distention, we performed semiquantitative scoring using the behavioral scale described by Al Chaer et al.[5] This consisted of visual observation of the animal's response to the
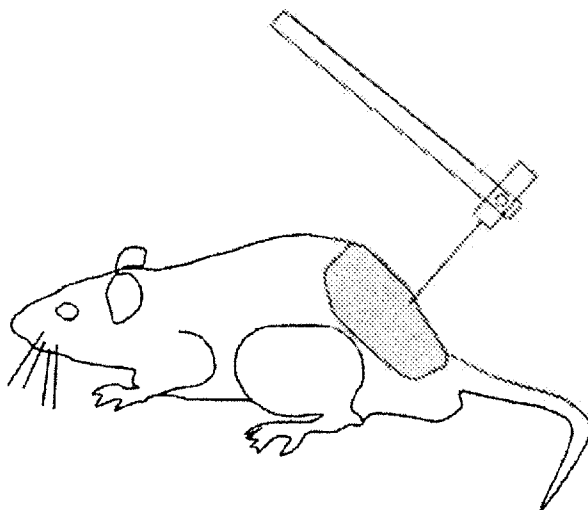
Figure 1. Schematic drawing illustrating the technique used to determine the referred lumbar cutaneous hyperalgesia (the *gray area* represents the lumbar shaving zone).

graded CRD by a blind observer and assignment of a score: 0, no behavioral response to CRD; 1, brief head movement followed by immobility; 2, contraction of abdominal muscles; 3, lifting of the abdomen; 4, body arching and lifting of the pelvic structures. Behavioral measurements were scored at several pressures (10, 20, 30, 40, 50, and 60 mm Hg) during distention.

Assessment of mechanical lumbar hyperalgesia.
Mechanical lumbar hyperalgesia, a marker of referred cutaneous pain, was measured by applying von Frey hairs to the lower back of rats (Figure 1). Rats were shaved on the lower back the day before the test. On the day of the test, they were acclimatized to a grid platform (30 × 45 × 25 cm) for 15 minutes and then calibrated von Frey hairs of increasing diameter were applied 5 times for 1 second, ranging from 2.1 to 72.3 mN (cutoff). The reaction thresholds (named "lumbar von Frey scores") corresponded to the force in mN of the von Frey hair that induced skin wrinkling in the lumbar area, followed or not by an avoidance behavior characterized by the rat escaping.

Assessment of Changes Observed in the Colon

Macroscopic study. Image analysis software (Image Tool, developed in the Department of Dental Diagnostic Science, University of Texas Health Science Center, San Antonio, TX) was used to obtain a macroscopic scoring of lesions. After removal, the bowel was laid on a white surface and a digital picture was taken. The picture was read using the software and converted into a gray scale. Lesion areas were determined over a length of 15 cm from the rectum by measuring surfaces that showed an increased gray level. A second evaluation used a slightly modified Morris scoring method[48]: 0, no damage; 1, localized hyperemia without

ulcers; 2, one site of ulceration with inflammation; 3, 2 or more major sites of ulceration and/or inflammation.

Histologic study. After removal, the fixed tissues (colonic tissue) were processed into paraffin, cut into 4-μm sections, and stained with HES or Giemsa colorations. Eosinophil and mast cell count within the lamina propria was systematically performed at a 400× magnification in 20 different areas. For each animal, results were expressed as the mean number of cells per area.

Determination of myeloperoxidase activity. Myeloperoxidase (MPO), a marker of polymorphonuclear neutrophil primary granules, was determined according to the method of Mazelin et al[24] but slightly modified. Briefly, a small section of colon (about 0.5–1 cm, taken at 7 cm from the anus) was rinsed with 0.1 mol/L cold phosphate buffer and suspended in hexadecyltrimethylammonium bromide buffer (0.5% wt/vol in 50 mmol/L phosphate buffer, pH 6.0; 50 mg of tissue/mL), a detergent that releases MPO from the primary granules. Intestinal tissue was homogenized using a Turrax (Ika-Werke, Staufen, Germany) and sonicated on ice for 30 seconds. The homogenate was then centrifuged at 1500g for 10 minutes at 4°C, freeze thawed 3 times, and centrifuged again. Supernatants were assayed by spectrophotometry for MPO activity and protein measurements. Each supernatant was mixed with 0.22% (vol/vol) aqueous guaiacol and 10 mmol/L phosphate buffer (pH 6.0). Three percent peroxide (H_2O_2) was added to start the reaction. Absorbance at 470 nm was determined with a spectrophotometer at 10-second intervals over 2 minutes. As a standard, MPO of human neutrophils (0.1 U/10 μL) was combined with the reaction buffer. The absorbance changes at 470 nm for 1 μmol peroxide/min were calculated from the standard curve, which equals 1 unit of MPO activity. Protein concentrations were determined by the bicinchoninic acid method[25] and MPO activity was expressed as unit MPO per gram of protein.

Neonatal Capsaicin Treatment

Time-dated pregnant Sprague–Dawley rats were housed with free access to food and water until delivery. Two-day-old male pups received capsaicin (50 mg/kg in 10/10/80 Tween 80/EtOH/saline [NaCl 0.9%]) or its vehicle subcutaneously. This technique is a well-established method of denervation, allowing destruction of more than 90% of C-fibers.[26] The effectiveness of capsaicin pretreatment to destroy C-fibers was assessed in adult rats (7–8 weeks) before any experiment using 3 different noxious stimuli: thermal (tail immersion test in a water bath at 46°C), mechanical (paw pressure test), and chemical (ocular application of 10 μL capsaicin in 0.1% EtOH) tests. Animals that responded positively to 2 of the 3 tests (delay >15 seconds for the tail immersion test, vocalization threshold >600 g for the paw pressure test, and no sign of eye scratching after ocular application of capsaicin) were selected for the experiment.

Experimental Protocols

All experiments were performed in a blind manner by the same experimenter using the block method, which consists of distributing one animal per treatment or control (saline-treated rats) in the same block. The number of blocks corresponds to the number of rats receiving each treatment. The order of treatments was randomized in each block. Different animals were used for each experimental series. All animals in a same block were treated in the same period of time. This procedure avoids any uncontrollable environmental influences.

Description of the model. *Effect of butyrate enemas on colonic sensitivity and on lumbar von Frey scores.* Five groups of 8 male rats were used. A group was instilled twice daily for 3 days with 1 mL saline and others with different concentrations of butyrate solution (8, 40, 200, and 1000 mmol/L). Lumbar von Frey scores and pressure-induced behavioral response to CRD were successively assessed on days 3, 6, 9, 12, 15, 18, 21, 24, and 27 after the first enema for the same animals.

Assessment of sex-dependent hypersensitivity was performed by instilling both male and female rats with saline or butyrate (200 mmol/L; n = 10–12 in each of the 4 groups). The same parameters were assessed on day 7 after the first enema.

Two other groups of 7–8 rats were used to confirm the colonic hypersensitivity using the method described by Al Chaer et al.[1] One group was instilled twice daily for 3 days with 1 mL saline and the other with a 200 mmol/L butyrate solution. Behavioral scoring was performed on day 7 after the first enema.

Effect of butyrate enemas on clinical parameters. Male rats were placed in individual metabolism cages with free access to food and water. They were allowed to acclimatize in these cages for 5 days. Two groups of 8 rats were used. The rats were instilled twice daily for 3 days with 1 mL saline or butyrate solution (200 mmol/L). The animals' body weight, the mass of wet and dry feces, and the volume of urine of each rat produced during the previous 24 hours were determined on days 0, 3, 6, 9, 12, and 15 after the first enema. The amount of food and water consumed was also determined.

The dry mass of the feces was determined after drying at 60°C for 48 hours. The water content (%) was then calculated using the following formula: $100 \times (1 - [Dry\ Mass/Wet\ Mass])$.

Effect of butyrate enemas on colonic mucosa. Three groups of 7 male rats were used. The first 2 groups were instilled twice daily for 3 days with 1 mL saline or butyrate solution (200 mmol/L). At day 3, the third group was instilled with a single intracolonic TNBS enema. This group was used as a positive control group with colonic inflammation.

At day 5, when inflammatory response was potentially maximal in the 2 models, all of the animals were killed by cervical dislocation. A midline laparotomy was performed, and the total colon was removed. The Morris score was determined, and a digital picture was taken for postmacroscopic analysis using Image Tool. The colon was then divided in half by a longitudinal cut. One part of the colon was frozen in liquid nitrogen and stored until MPO activity assay, and the other part was immersion fixed in 10% formalin for histologic study.

Pharmacologic validation of the model. All the groups of 8 rats were instilled with 1 mL butyrate solution (200 mmol/L) twice daily for 3 days. Seven days after the beginning of butyrate instillations, 3 experimental series corresponding to the different treatments were performed. Animals were treated with morphine (0.03, 0.1, 0.3, 1, 3, and 10 mg/kg subcutaneously), U50488H (0.3, 1, 3, and 10 mg/kg intraperitoneally), or trimebutine (3, 10, 30, and 100 mg/kg intraperitoneally). The von Frey and CRD tests were performed respectively in the same animals 25 and 35 minutes after the injections. Each control group was injected with saline.

Determination of C-fiber involvement. *Effect of neonatal capsaicin treatment on butyrate-induced colonic hypersensitivity and mechanical lumbar hyperalgesia.* Four experimental groups of 10 male rats were used. Rats were pretreated with capsaicin (and selected after noxious tests) and instilled twice daily for 3 days with 1 mL saline or butyrate solution (200 mmol/L). Mechanical lumbar hyperalgesia and pressure-induced behavioral response to CRD were determined on day 7 after the first enema for the same animals.

Effect of CGRP$_{8-37}$, a CGRP antagonist, and L733060, a selective NK1 receptor antagonist, on butyrate-induced colonic hypersensitivity and mechanical lumbar hyperalgesia. Four experimental groups of 8 rats were used. All of the groups were instilled with 1 mL butyrate solution (200 mmol/L) twice daily for 3 days. Seven days after the beginning of butyrate instillations, they received an intravenous injection of saline, CGRP$_{8-37}$ (20 μg/kg), or L733060 (1 and 3 mg/kg) as described by Gschossmann et al[19] and Rupniak et al,[27] respectively, 10 minutes before the CRD test. The same injection protocol was used 10 minutes before von Frey testing on different animals.

Expression of Results and Statistical Analysis

Results are expressed as mean ± SEM of raw data. Results of the CRD testing were analyzed using a one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test to compare several treatments or by a 2-way ANOVA followed by a Student–Newman–Keuls post-hoc test when several treatments were compared over the same time course. von Frey scores were analyzed using nonparametric Kruskal–Wallis ANOVA on ranks followed by a Dunn's test when several treatments were compared over the same time course or by a Mann–Whitney rank sum test to compare 2 groups. Differences were considered significant at $P < .05$.

Chemicals

The following agents were used in this study: acepromazine (Vetoquinol, Lure, France); ketamine (Panpharma, Luitré Fougères, France); n-sodium salt butyrate, TNBS, Evan's blue, acetone, sodium sulfate, hexadecyltri-
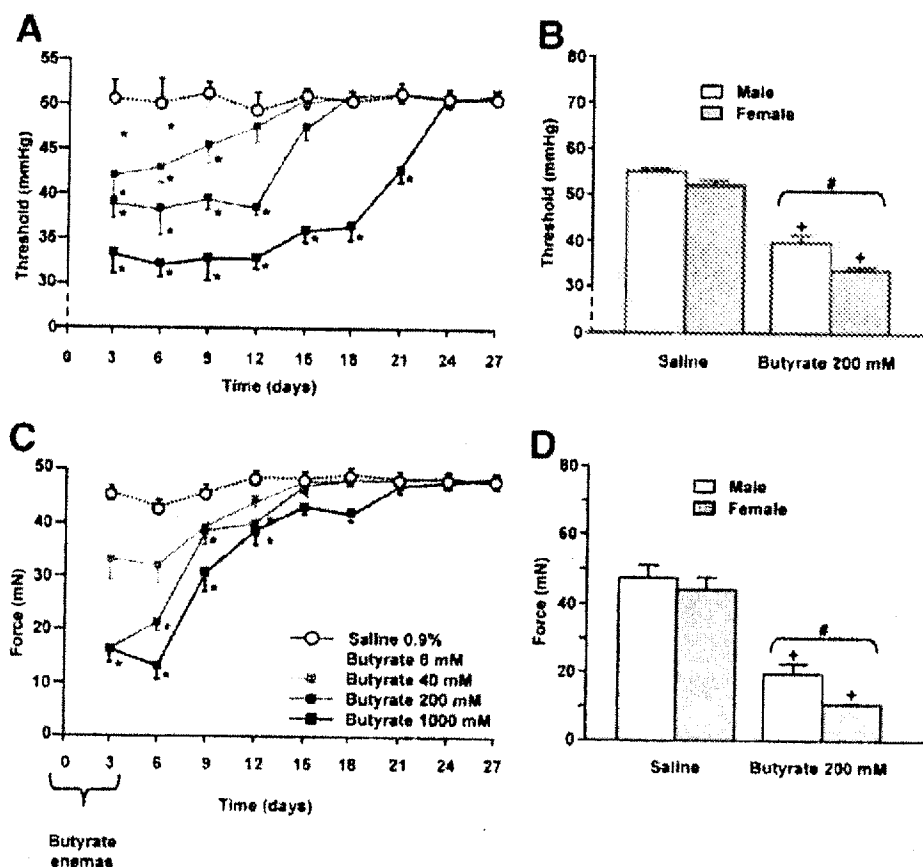
Figure 2. Effect of 6 enemas (twice daily) of 1 mL saline solution or of 8, 40, 200, or 1000 mmol/L butyrate solution (A) on the pressure thresholds inducing specific behavior following CRD and (C) on forces exerted by the lumbar application of von Frey hairs inducing a reaction (lumbar von Frey scores) in rats. These parameters were monitored every 3 days up to 27 days. $n = 8$ per group. Effect of 6 enemas (twice daily) of 1 mL saline or 200 mmol/L butyrate solution (B) on the pressure thresholds and (D) on lumbar von Frey scores in male and female rats. n ~ 10–12 per group. *$P < .05$ versus saline-instilled group. (A) 2-way ANOVA followed by a Student–Newman–Keuls post-hoc test, or (C) Kruskal–Wallis one-way ANOVA on ranks followed by a Dunn's post-hoc test. '$P < .05$ versus saline-instilled group. (B) one-way ANOVA followed by a Bonferroni post-hoc test, or (D) Mann–Whitney rank sum test. #$P < .05$ versus female butyrate-instilled group. (B) one-way ANOVA followed by a Bonferroni post hoc test, or (D) Mann–Whitney rank sum test.

methylammonium bromide buffer, phosphate buffer, ibuprofen, naloxone, U50488H, MPO of human neutrophils, guaiacol, capsaicin, L733060, and CGRP$_{x..,}$ (Sigma Aldrich, Lyon, France); morphine (Cooperation Pharmaceutique Française); bicinchoninic acid (Interchim, Paris, France); and trimebutine (Debridat; Pfizer, Paris, France).

Results

Description of the Model

Effect of butyrate enemas on colonic sensitivity and on lumbar von Frey scores. Butyrate induced a significant concentration-dependent decrease of thresholds to CRD (Figure 2A) and of von Frey scores (Figure 2C) in 100% of butyrate-instilled animals, while scores for saline-treated male rats remained stable throughout the experiment (50.6 ± 2.1 mm Hg

and 45.7 ± 1.3 mN for CRD and von Frey scores, respectively). A significant decrease in CRD thresholds was observed for the lowest butyrate concentration (8 mmol/L) at days 3 and 6 after the start of butyrate enemas. The intensity and duration of these changes increased with the butyrate concentrations. For the 200-mmol/L butyrate concentration, selected for further investigations, the maximal decrease was obtained at day 6 (~26.1% ± 3.1%) and this decrease was almost constant until day 12. As observed in Figure 2C, the concentration-dependent decrease in von Frey scores was marked (maximal decrease for 200 mmol/L butyrate, ~64.3% ± 5.6%) but shorter than for the decrease in CRD thresholds. For instance, these thresholds were almost stable for 18 days after the start of 1000 mmol/L butyrate enemas while von Frey
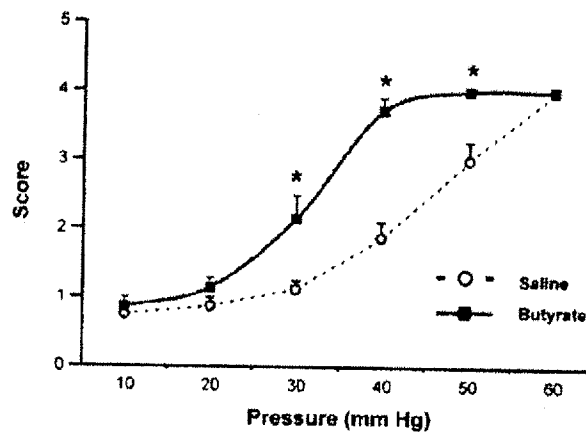
Figure 3. Effect of 6 enemas (twice daily) of 1 mL saline solution or a 200 mmol/L butyrate solution on pain score using the method of Al Chaer et al measured at several CRD pressures (every 10 mm Hg from 0 to 60 mm Hg). n = 7-8 per group. *P < .05 versus saline-instilled group. Mann–Whitney rank sum test.

scores started to return to normal values after day 6, although they remained significantly decreased for 12 days.

All of these data account for a colonic hypersensitivity and a mechanical lumbar hyperalgesia that, moreover, were significantly more marked in female than in male rats (see Figure 2B and D, respectively). Decreases in scores were −27.7% ± 3.06% and −35.5% ± 1.6% for CRD thresholds and −62.9% ± 6.8% and −77.0% ± 0.9% for von Frey scores (in male and female rats, respectively).

The complementary study confirmed the hypersensitivity of animals instilled with butyrate (200 mmol/L). In fact, the curve of relationship between the pain score obtained and pressure shifted to the left in butyrate-instilled animals, showing a clear reduction in pressure that induced specific pain behavior (Figure 3). In butyrate-instilled animals, there was a significant increase in scores for pressures between 30 and 50 mm Hg. At 60 mm Hg, all animals, regardless of treatment, exhibited a marked pain position with the maximal score of 4.

Effect of butyrate enemas on clinical parameters. As shown in Table 1, butyrate enemas did not modify the evolution in animals' body weight. Similarly, none of the other parameters (food and water intake, volume of urine, fecal dry mass, and water content) were different between butyrate-instilled and saline-instilled animals. The lack of modification in feces suggests that there was no change in the bowel transit.

Effect of butyrate enemas on colonic mucosa. Studies of the colonic mucosa did not show any modification in saline- and butyrate-treated animals, contrary to the positive controls (TNBS-treated rats), which showed clear inflammation-induced mucosal damage (Figure 4 and Table 2). Thus, the macroscopic damage scores evaluated by the Morris scoring or Image Tool software methods did not differ between saline- and butyrate-instilled animals, while there was a significant and important increase in these scores for TNBS-treated rats. The microscopic organization of mucosa was totally destroyed in TNBS-treated rats (Figure 4C), while the

Table 1. Effect of Butyrate Enemas on Clinical Parameters

	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Weight of rats (g)						
Butyrate	216 ± 3	242 ± 3	263 ± 4	283 ± 5	306 ± 6	323 ± 7
Saline	234 ± 5	252 ± 6	268 ± 7	289 ± 7	304 ± 8	321 ± 8
Food intake (g)/weight of rats (g)						
Butyrate	0.11 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
Saline	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	0.08 ± 0.02
Water intake (mL)/weight of rats (g)						
Butyrate	0.11 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.09 ± 0.01
Saline	0.10 ± 0.02	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.01
Urine volume (mL)/weight of rats (g)						
Butyrate	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Saline	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Fecal dry mass (g)/weight of rats (g)						
Butyrate	17 ± 2	18 ± 1	15 ± 1	15 ± 1	15 ± 1	12 ± 1
Saline	16 ± 1	14 ± 1	16 ± 1	21 ± 1	15 ± 1	14 ± 1
Water content (%)/weight of rats (g)						
Butyrate	44 ± 2	50 ± 1	46 ± 2	43 ± 1	42 ± 2	45 ± 2
Saline	46 ± 6	55 ± 3	50 ± 2	46 ± 1	47 ± 1	46 ± 2

NOTE. The rats received enemas of saline or sodium butyrate (200 mmol/L) twice daily for 3 days from days 1 to 3. Results are expressed as means +/− SEM. n = 8 in each group.
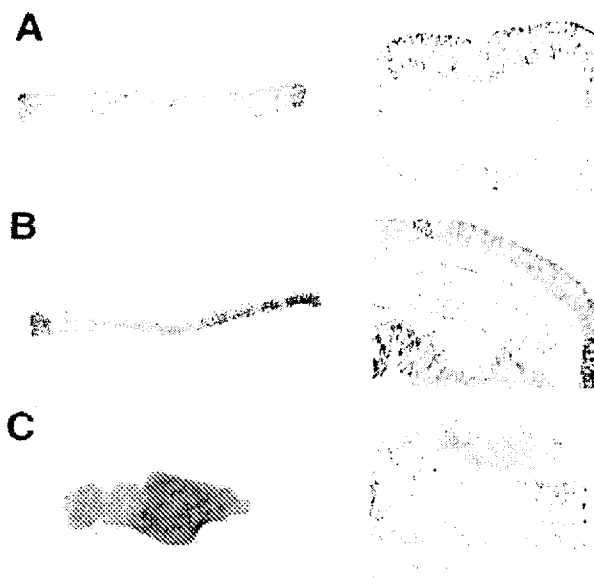
A

B

C

Figure 4. Macroscopic and microscopic (histologic HES staining; original magnification 100×) images of the colon taken from rats receiving (A) 6 enemas (twice daily) of 1 mL saline solution, (B) 6 enemas (twice daily) of 200 mmol/L butyrate solution, or (C) one enema of TNBS solution. The bowel of TNBS-treated animals presents a complete destruction of the epithelial architecture, with an almost complete loss of crypts, and epithelial integrity. Edema and intense inflammation are present in all layers. The colons of butyrate-treated animals are normal. n = 7 per group.

different layers remained similar in control (Figure 4A) and butyrate-instilled rats (Figure 4B). Moreover, in our model, there was no increase in MPO activity compared with the TNBS model. The histologic study with quantification of mast cells and eosinophils showed an increase in these cells in the TNBS model that was significant for eosinophils, contrary to butyrate-instilled animals.

Pharmacologic Validation of the Model

Morphine induced a dose-dependent decrease of the colonic hypersensitivity assessed by the CRD test and of the mechanical lumbar hyperalgesia determined by the von Frey test (Figure 5A). The effect of morphine on CRD thresholds and lumbar von Frey scores was statistically significant from the low dose of 0.3 mg/kg subcutaneously and the dose of 3 mg/kg subcutaneously, respectively. The highest doses (>1 mg/kg) increased scores over the basal values of rats instilled with saline enemas (50.6 ± 2.1 mm Hg and 45.7 ± 1.3 mN, respectively; see Figure 1) and consequently had an antinociceptive effect.

As was the case for morphine, U50488H also induced a significant effect on the 2 pain parameters (Figure 5B). The first active dose was 1 mg/kg intraperitoneally on

CRD thresholds, with a maximal effect lower than that observed with morphine, and only the highest dose (10 mg/kg intraperitoneally) significantly increased lumbar von Frey scores.

Trimebutine induced an antihyperalgesic effect, although it was lower. Trimebutine significantly increased the CRD threshold from the 30-mg/kg intraperitoneal dose. Lumbar von Frey scores were increased, but the variation was not significant (Figure 5C).

Determination of C-Fiber Involvement

Effect of neonatal capsaicin treatment on butyrate-induced colonic hypersensitivity and mechanical lumbar hyperalgesia. Capsaicin-pretreated rats instilled with butyrate solution had a significant increase in their CRD thresholds and lumbar von Frey scores compared with corresponding vehicle-pretreated animals (Figure 6A and B). This significant increase in both parameters was also observed in saline-instilled animals. Furthermore, no significant difference was observed between saline- and butyrate-treated groups in capsaicin-pretreated rats.

Effect of CGRP$_{8-37}$, a CGRP antagonist, and L733060, a selective NK1 receptor antagonist, on butyrate-induced colonic hypersensitivity and mechanical lumbar hyperalgesia. CGRP$_{8-37}$ (20 μg/kg injected intravenously; Figure 7A and B) significantly increased the CRD thresholds and brought them back to those of healthy animals. Using the same administration routes, the CGRP receptor antagonist reduced mechanical lumbar hyperalgesia, but the effect was not statistically significant. In contrast, intravenous injection of the selective NK1 receptor antagonist L733060 did not modify CRD thresholds or lumbar von Frey scores (Figure 7A and B).

Table 2. Effect of Butyrate and TNBS Enemas on Colonic Mucosa

	Saline	Butyrate	TNBS
Macroscopic damage score			
Morris score	0	0	2.7 ± 0.3[a]
Image Tool score (%)	6 ± 3	4 ± 3	42 ± 5[a]
MPO activity (IU/g protein)	ND	ND	7490 ± 1015[a]
Histologic study			
Mast cells	0.3 ± 0.1	0.2 ± 0.1	1 ± 0.3
Eosinophils	5.3 ± 2	3.5 ± 0.5	29.9 ± 4[a]

NOTE. Analyses were performed 2 days after the end of saline or butyrate (200 mmol/L) enemas (twice daily for 3 days) or 2 days after intracolonic injection of TNBS (50 mg/kg). Results are expressed as means +/− SEM. n = 7 in each group.
ND, not detected.
[a]$p < .05$ versus saline-treated group, one-way ANOVA followed by a Student-Newman-Keuls post-hoc test.
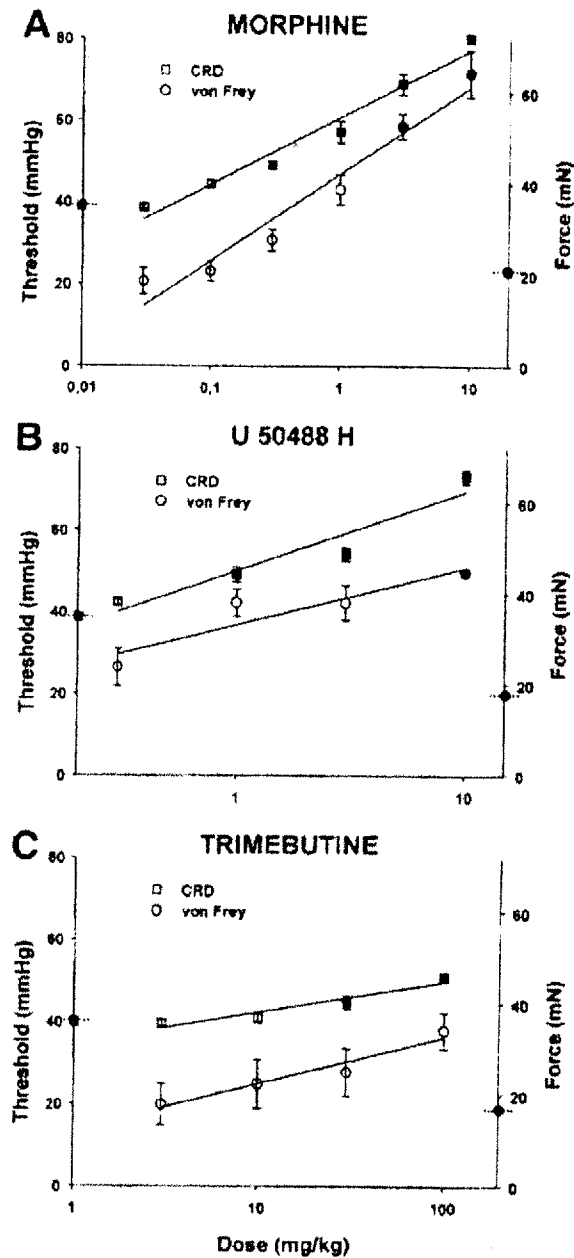
Figure 5. Effect of (A) morphine (0.03, 0.1, 0.3, 1, 3, and 10 mg/kg subcutaneously), (B) U50448H (0.3, 1, 3, and 10 mg/kg intraperitoneally), and (C) trimebutine (3, 10, 30, and 100 mg/kg, intraperitoneally) on the colonic hypersensitivity and referred lumbar mechanical hyperalgesia induced by 6 enemas (twice daily) containing 1 mL of a 200 mmol/L butyrate solution. Colonic hypersensitivity and lumbar mechanical hyperalgesia were determined 7 days after the beginning of butyrate enemas using the CRD test (squares) and the von Frey test (circles). Scores of butyrate-treated animals receiving saline (0.9% NaCl) are presented as gray squares (for the CRD test) and gray circles (for the von Frey test) on their respective y-axes. n = 8 in each group. Black squares or black circles show a significant difference (P < .05) versus the control group (butyrate-instilled rats treated with saline in the same conditions); one-way ANOVA followed by a Bonferroni post-hoc test (CRD test; squares) or Kruskal–Wallis one-way ANOVA on ranks followed by a Dunn's post-hoc test (von Frey scores; circles).

Discussion

This study clearly shows that colonic butyrate enemas induce a hypersensitivity to CRD characterized (1) by a decrease in pressure that induced a clearly visible abdominal contraction (pain behavioral response), (2) by a shift to the left of the curve of the scores of abdominal pain (method of Al Chaer et al[5]) induced by increasing distention pressures, and (3) by a referred cutaneous lumbar hyperalgesia assessed using the von Frey test. This result, together with the nature of the inducing factor, the observed sex difference, the lack of colonic inflammation, and the pharmacologic sensitivity, makes this a novel and relevant model for colonic hypersensitivity as found in patients with IBS.
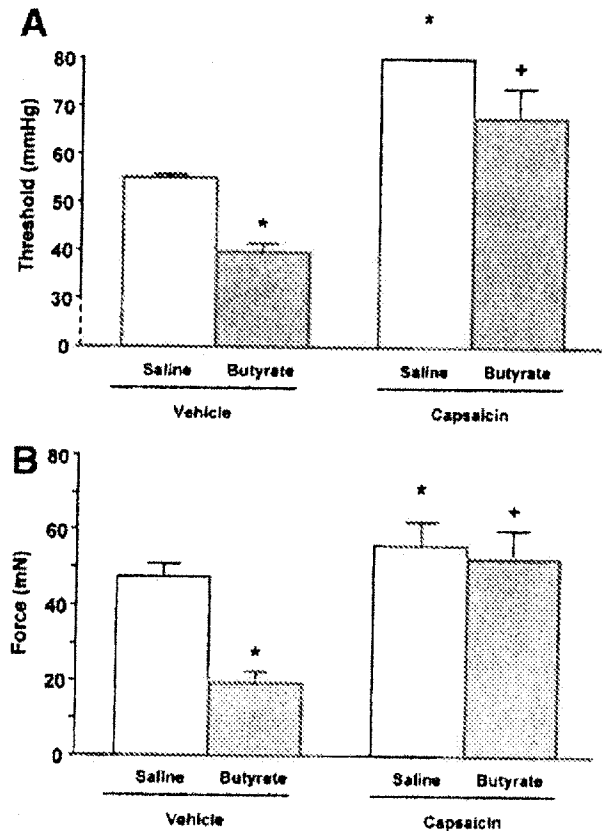

Figure 6. Effect of 6 enemas (twice daily) of 1 mL saline or 200 mmol/L butyrate solution (A) on the pressure thresholds inducing specific behavior following CRD and (B) on lumbar von Frey scores in male rats. Colonic hypersensitivity and lumbar mechanical hyperalgesia were determined 7 days after the beginning of butyrate instillations using the CRD test and the von Frey test. Animals were pretreated at 2 days of age either with vehicle or with capsaicin. n = 10 in each group. *P < .05 versus vehicle-treated group instilled with saline. +P < .05 versus vehicle-treated group instilled with butyrate. One-way ANOVA followed by (A) Bonferroni post-hoc test or (B) Mann–Whitney rank sum test.
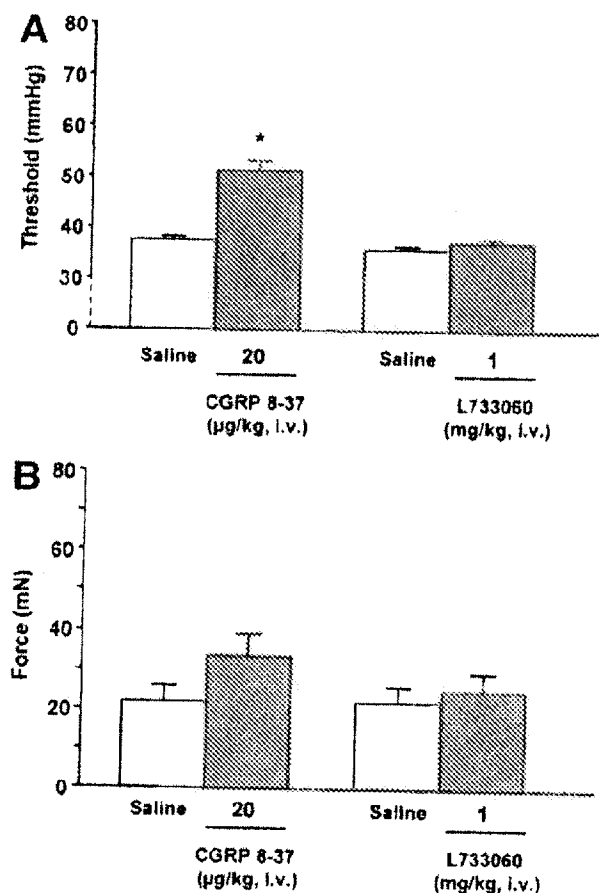
Figure 7. Effect of a CGRP receptor antagonist ($CGRP_{8-37}$) and an NK1 receptor antagonist (L733060) on the (A) colonic hypersensitivity and (B) referred lumbar mechanical hyperalgesia induced by 6 enemas (twice daily) of 1 mL of 200 mmol/L butyrate solution. Colonic hypersensitivity and lumbar mechanical hyperalgesia were determined 7 days after the beginning of butyrate instillation using the CRD test and the von Frey test, respectively. n = 8 in each group. *$P < .05$ versus respective control group (treated with saline), one-way ANOVA followed by (A) Bonferroni post-hoc test or (B) Mann–Whitney rank sum test.

Description of the Model

The nature of butyrate, which induces hypersensitivity, makes this model particularly relevant. Indeed, most physicians, scientists, and patients are convinced that functional symptoms of IBS are impacted to some degree by dietary factors,[28] and there are data suggesting that fibers (and their metabolites) could be one of them. Thus, increased colonic levels of butyrate have been observed in patients with IBS,[9] and Francis and Whorwell[29] showed that wheat bran worsened the IBS symptoms of 55% of 100 secondary care patients with IBS. This is why Dapoigny et al[30] concluded in their review that fibers, commonly believed to be beneficial in IBS,

should induce or increase symptoms in patients with IBS and must therefore be used with care.

The second major interest of this model is the determination of a colonic hypersensitivity as found in patients with IBS after CRD,[5] combined with a referred cutaneous lumbar hyperalgesia. To our knowledge, this is the first time that a referred mechanical hyperalgesia has been observed in conscious animals with sustained visceral hypersensitivity. This is in line with interesting results reported by Zhang et al[31] demonstrating a mechanical cutaneous hyperalgesia following CRD in anesthetized rats. Such a combination has also been observed in patients with IBS[32] who, during CRD, present rectal hypersensitivity and cutaneous allodynia to thermal stimulation restricted to the lumbosacral dermatomes. Colonic hypersensitivity plateaued for 12 days after treatment with 200 and 1000 mmol/L, whereas hyperalgesia plateaued for 6 days. The shorter duration of cutaneous referred hyperalgesia could be explained by the fact that referred visceral hypersensitivity has different extents and time courses in superficial (skin, subcutis) versus deep somatic structures (muscle) of the body wall. It has been shown in human subjects[34,36] and in animals[35,38] that referred visceral hyperalgesia is particularly pronounced in the muscles, with a long duration (normally it outlasts the duration of the spontaneous pain and sometimes even the presence of the primary algogenic focus in the internal organ). In contrast, referred hypersensitivity, which is not always present in superficial tissues, is less pronounced and of a lesser duration than deep somatic hypersensitivity and is capable of reverting despite the persistence of the visceral focus.[39] In our animal model, we tested the hypersensitivity in the skin of the referred pain area; it is therefore not surprising that duration was less pronounced than that for the visceral hypersensitivity itself. However, the simple fact that superficial cutaneous hypersensitivity developed as a consequence of the butyrate-induced colonic stimulation that we performed shows the robustness of the model used; only in cases of strong nociceptive stimulation of viscera does cutaneous referred hypersensitivity develop.[40]

A third interesting feature of the model is that the colonic hypersensitivity and the referred cutaneous hyperalgesia were statistically greater in female than in male rats. This supports the greater sensitivity in female rats described by Rosztoczy et al[41] in a model of rectal hypersensitivity of adult rats induced by maternal deprivation. This is also in agreement with clinical observations showing that patients with IBS are mostly female[42,43] and that female patients with IBS are more sensitive to CRD than male patients.[5,44]

Another feature is that the macroscopic and microscopic control of the integrity of the colonic mucosa failed to show any alteration in butyrate-instilled rats. This observation is in line with histologic and biochemical measurements showing that there was no difference (the same low number of mast cells and eosinophils, same undetectable MPO activity) between control and butyrate-treated rats, contrary to our positive controls (TNBS-instilled animals), which presented significant mucosal impairment. This is an important point for concluding that we obtained colonic hypersensitivity without any detectable structural or biochemical abnormality of the mucosa, as usually observed in patients with IBS.[10] This makes our model relevant and original when compared with some others. For instance, Al Chaer et al[5] induced a colonic hypersensitivity in adult rats following mechanical or chemical colonic irritation during the postnatal period. Diop et al,[7] in their model, induced a distant colonic hypersensitivity (distal part of the colon) after having induced an inflammation in the proximal part with TNBS.

Finally, our model failed to induce any alteration in the clinical parameters of food intake, weight, and behavior, suggesting a good general state of the animals. We also observed that there was no modification of bowel transit in butyrate-instilled animals.

Pharmacologic Sensitivity

The pharmacologic investigations showed that our model presented good sensitivity to pharmacologic manipulations when compared with the pharmacotherapy of patients with IBS. The 2 opioid drugs morphine and U50488H induced a dose-dependent, antinociceptive effect as measured by both the CRD and the von Frey testing. These results are in agreement with several data showing the efficacy of μ and κ opioid receptor agonists in acute colonic pain tests in healthy animals,[15] in subchronic or chronic colonic pain models,[7,10, 18] and in patients with IBS.[19,50] Another drug, trimebutine, which has been shown to be effective in patients with IBS,[51] significantly reduced the colonic hypersensitivity.

Peptidergic C-Fiber Involvement

In the experiment performed in animals pretreated with capsaicin, we clearly observed that the induced destruction of C-fibers, validated by 3 acute pain tests applied to each animal, strongly increased the CRD thresholds in saline- or butyrate-instilled rats to higher levels than those of saline-instilled animals pretreated with vehicle. This result confirms several data showing reduced nociception following pretreatment with capsaicin in several pain contexts,[52,54] including visceral in-

flammatory pain models,[54,55] or in the TNBS-induced distant colonic hypersensitivity model.[56] Butyrate-induced referred cutaneous hyperalgesia was also totally reversed by the neonatal pretreatment with capsaicin. Taken together, these results suggest the involvement of C-fibers in the butyrate-induced hypersensitivity. However, while neonatal pretreatment with capsaicin can destroy afferent C-fibers, it could also induce changes in ascending pathways and descending modulatory influence on spinal nociceptive input. Neonatal treatment with capsaicin results in a selective elimination of neurons expressing the VR1 capsaicin receptor (TRPV1).[57] There is ample evidence that neonatal treatment with capsaicin, as described by Jancso et al,[58] results in a selective elimination of C-fiber nociceptive afferents that express this receptor.[57] However, the effect of neonatal capsaicin pretreatment can also affect modulation of pain at the spinal level. Thus, Zhuo and Gebhart[59] have shown that neonatal capsaicin treatment could lead to modified modulation of the rostral medial medulla on spinal nociceptive tail-flick reflex. TRPV1 is also widely recovered in the brain,[60] notably in some central regions associated with nociception such as the periaqueductal grey and in a number of thalamic nuclei.[61] However, the exact functional role of these neurons remains largely unknown,[62,63] and they do not appear to be destroyed by neonatal treatment with capsaicin.[60]

Beyond the transmission of peripheral nerve influx to the spinal cord, capsaicin-sensitive peptidergic C-fibers are of major importance in the generation of neurogenic inflammation and peripheral nerve sensitization due to retrograde release of neuropeptides.[64] We can suspect such a mechanism in our model due to the hypersensitivity observed and its reduction by CGRP₈₋₃₇, a CGRP receptor antagonist, which fails to cross the blood-brain barrier.[19] The positive effect of the CGRP antagonist is not surprising because a lot of afferent C-fibers contain CGRP, which has been shown to be pronociceptive at the visceral level.[51,55,65] Moreover, CGRP antagonists have been shown to be effective in several visceral pain models.[19,51,55,66] The involvement of CGRP in our experimental model of colonic hypersensitivity is also indirectly confirmed by the fact that morphine and U50488H were effective in reducing colonic hypersensitivity. Friese et al[51] reported that μ and κ opioid agonists can reduce CGRP-induced abdominal contractions.

While CGRP would appear to be involved in the apparition of the colonic hypersensitivity, the lack of effect of an NK1 receptor antagonist on the pain thresholds suggests that substance P is not. In fact, the effects of NK1 antagonists in visceral pain models are contra-

dictory. Some studies show the efficacy of systemically administered NK1 antagonists in models of colonic hypersensitivity,[67-70] while others fail to do so.[47,65,71] It has also been suggested by Sanger[72] that NK1 receptors could be involved in mucosal inflammatory processes but not in pain processes, as observed by Amann et al,[73] who showed that SR140333, another NK1 receptor antagonist, reduces neurogenic inflammation without modifying acute chemonociception or thermonociception in a somatic model of sustained pain in rats.

To conclude, we propose that repeated colonic instillations of butyrate could be used as a new relevant animal model of colonic hypersensitivity as observed in patients with IBS. This model is in line with dietary factors that could be involved in functional bowel disorders and raises questions about the therapeutic benefit of fibers or butyrate in patients with IBS. It could be used to determine the efficacy of analgesic compounds on noninflammatory colonic hypersensitivity but also on referred mechanical cutaneous lumbar hyperalgesia assessed on the same animals. The preliminary studies performed in this study on the mechanisms involved in this hypersensitivity suggest an involvement of peptidergic C-fibers. The activation of these primary afferent fibers could be followed by a stimulation of peripheral CGRP receptors, but not NK1 receptors, due to the retrograde release of the peptide. Accordingly, we suggest that CRGP receptors may prove very interesting targets for the treatment of abdominal pain in patients with IBS.

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